

From the Department of Genetics, University of California, Davis  
COMPARATIVE STUDIES OF CHROMOSOME PAIRING  
IN NATURAL AND INDUCED TETRAPLOID DACTYLIS\*

By

GILBERT D. MCCOLLUM

With 15 Figures in the Text

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A. Introduction and Review

I. Origin of the Tetraploids

The forage grass, *Dactylis glomerata* L. (orchard grass or cocksfoot), frequently cited as a successful naturally-occurring autotetraploid (MÜNTZING 1936, 1937; MYERS 1947; ATWOOD 1947; STEBBINS 1947, 1950; CLAUSEN, KECK and HIESEY 1945; SCHWANITZ 1954; STEBBINS and ZOHARY 1958) was suspected by MÜNTZING (1937) to have originated by chromosome doubling of *Dactylis aschersoniana*, the only diploid *Dactylis* known twenty years ago. The differences between diploid *aschersoniana* and tetraploid *glomerata*, especially in vigor, type of panicle and leaf color, MÜNTZING believed to be caused directly by chromosome doubling rather than by differences in specific genes. However, he qualified his opinion by writing: "It is not probable that the diploid *Dactylis* types now growing in South Sweden, Denmark and Central Europe are the actual progenitors of *D. glomerata*, but nevertheless, the present *D. aschersoniana* must be very closely related to the original forms of *glomerata*."

CLAUSEN, KECK and HIESEY (1945, pp. 137—138) suggested that other forms of *Dactylis*, e.g. *abbreviata*, or the Mediterranean *hispanica*, might be diploid and have contributed to the origin of *glomerata*. STEBBINS (1947) called *D. glomerata* an "intervarietal autopolyploid" and proposed *D. hispanica* or *D. juncinella* as responsible for the morphological differences between *aschersoniana* and *glomerata*. Then MYERS (1948) reported a diploid from Iran, now identified as ssp. *woronowii* (cf. STEBBINS and ZOHARY 1958), which was interfertile with *aschersoniana*. The  $F_1$  hybrid resembled many plants of *glomerata*.

Additional diploids are now known (ZOHARY 1955; WEIBULL, cited by MÜNTZING 1956; STEBBINS 1956b; STEBBINS and ZOHARY 1958). STEBBINS and ZOHARY (1958) describe eleven diploids which may have contributed to the variation at the tetraploid level and conclude that the genus *Dactylis* consists of a single species with several morphologically and ecologically distinct but interfertile diploid subspecies. Most of the diploids are limited in distribution to small areas near the periphery of a much greater area of tetraploid distribution. The widespread and variable tetraploids, usually intermediate between two or more diploid subspecies in morphology and habitat, are thought to have arisen initially from hybridizations between diploids, followed by chromosome doubling of the hybrids, i.e. inter-subspecific autotetraploidy. That some tetraploids were difficult to distinguish morphologically from diploids suggests that "pure" as well as inter-subspecific autotetraploidy is involved.

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Independently of STEBBINS and ZOHARY, MÜNTZING (1956) cited unpublished work of WEIBULL to characterize the *Dactylis* complex in a way similar to the one reviewed above. He suggested two natural units, diploid and tetraploid. The different diploids should be considered subspecies or ecotypes, and *D. glomerata*, "an autotetraploid probably arising from crosses between different subunits of the same diploid species". However, other possible events, for example chromosome doubling in hybrid generations later than the  $F_1$  (SCHWANITZ 1954, p. 489) and hybridization between different tetraploids or between diploids and tetraploids (cf. MÜNTZING 1937; STEBBINS and ZOHARY 1958; HANSON and CARNAHAN 1956, p. 62) could make the initial origins less clear.

HANSON and CARNAHAN (1956) discuss another kind of evidence that *D. glomerata* is not a pure autotetraploid, namely that inbreeding depression in tetraploid orchard grass approaches the amount expected in a diploid species like maize (cf. KALTON, SMIT and LEFFEL 1952). On this evidence and the suspected involvement of two closely related diploids in the origin of the tetraploid, HANSON and CARNAHAN suggested that the tetraploid could be a segmental allopolyploid and as such might show both tetrasomic and disomic patterns of inheritance. Reference should be made to STEBBINS (1947, 1950) for discussions of this type of polyploid.

## II. Chromosome Cytology of the Tetraploids

A few genetic studies have shown tetrasomic inheritance for some chlorophyll defects (MYERS 1941; BRIX and QUADT 1953), but the main evidence for autotetraploidy is cytological. Although CHURCH (1929) reported 14 bivalents, 28 univalents, or some combination of bivalents and univalents at diakinesis, later workers have consistently reported quadrivalents to be frequent in *Dactylis*. MÜNTZING (1933, 1937) made detailed studies of meiosis in tetraploids, reporting mean numbers of quadrivalents in a cultivated variety, Skandia II, as 3.5 and 3.48 quadrivalents per cell at diakinesis and first metaphase, and in a wild biotype from Altai, Central Siberia, as 3.0 and 2.8 quadrivalents per cell. He later studied two "haploid" twins obtained from *glomerata*, one of which formed 7 bivalents (MÜNTZING 1943). Meiosis of several tetraploids naturalized or cultivated in northeastern United States and of some Foreign Plant Introductions was studied in great detail by MYERS and HILL (MYERS and HILL 1940, 1942, 1943; MYERS 1943), making *Dactylis glomerata* the "most completely investigated" of forage grasses in providing information on polyploidy (MYERS 1947). Discussions of this series of papers are to be found in MYERS (1945, 1947) and ATWOOD (1947).

MYERS and HILL (1940) reported mean numbers of quadrivalents in three plants to be 3.3, 3.8 and 4.2 per cell. For twenty plants listed in a later paper (1942), 2.42 in one plant and from 3.30 to 4.39 quadrivalents in the other nineteen were obtained. Eighty-three inbred plants, the progeny obtained by self-pollinating eight of the twenty plants mentioned above, had means of from 2.62 to 4.91 quadrivalents, whereas the values for the eight parent plants in the same season ranged from 3.00 to 4.09 (MYERS and HILL 1943). A range of means from 3.0 to 4.3 was reported by MYERS (1943). An extension of this work to hexaploid *Dactylis* was made by HANSON and HILL (1953). Chromosome pairing in other tetraploids was studied by ZOHARY (1955) who reported the mean numbers of quadrivalents as 3.18, 3.28 and 3.64 per cell in tetraploids from Ankara, Turkey; Malaga, Spain (*hispanica* type); and Sintra, Portugal (*maritima* type), respectively.

When large numbers of cells are examined, the frequency distributions of numbers of cells with 0, 1, 2, ... 7 quadrivalents are unimodal, and chromosome configurations consist almost entirely of quadrivalents and bivalents; only RANCKEN (1934, cited by MÜNTZING 1937) reported trivalents and univalents

to be frequent. Distribution of chromosomes at first anaphase has been described as regular, although some irregularity is indicated by MÜNTZING's finding (1937) that from 4 to 26 percent of the offspring of single isolated and probably euploid plants had somatic chromosome numbers of 26, 27, 29 or 30. Likewise MYERS and HILL (1940) found 41 percent aneuploids among 116 plants, some of whose parents however were probably aneuploid also. MÜNTZING attributed the irregularities in chromosome distribution, which must result in lowered fertility and aneuploid offspring, to the frequent occurrence of quadrivalents. However, very extensive and detailed analyses by MYERS and HILL of quadrivalents, unpaired chromosomes at first metaphase, lagging and dividing univalents at first anaphase, micronuclei in young microspores, seed set, and correlations among these made it clear that the presence of quadrivalents had little influence on irregularities at later stages and on fertility (MYERS and HILL 1942, 1943; MYERS 1943). The incidence of univalents at first metaphase, positively correlated with lagging and dividing univalents at first anaphase and micronuclei at quartet stage, and negatively correlated with seed set, was regarded by these workers as the main cytologically-observable cause of aneuploid gametes and decreased fertility. A high negative correlation between quadrivalent and univalent frequencies seemed to come mainly through a common relationship with chiasma frequency, which was positively correlated with quadrivalents and negatively correlated with univalents. The same interrelationships were found in hexaploid *Dactylis* (HANSON and HILL 1953).

In both hexaploids and tetraploids, there were significant differences between plants in most of the features of meiosis studied. Inbreeding lowered the chiasma frequency in some families while increasing the univalent frequency by two or three times in most. Inbreeding increased the frequency of quadrivalents in some families but had no effect in others. From an experiment which could assess the importance of environmental factors, MYERS (1943) concluded that the significant differences between plants in numbers of quadrivalents were dependent only slightly on environmental conditions, but to a greater extent on chiasma frequency and other factors, among which *chromosomal differentiation* was suggested.

Little work has been published on colchicine-induced tetraploids of *Dactylis*. MYERS (1948) reported that induced tetraploid *aschersoniana* resembled natural *D. glomerata* cytologically and produced fertile hybrids with it. NIELSEN (1952), citing MYERS (unpublished), states that induced tetraploids from the hybrid between *aschersoniana* and *woronowii* are similar to *D. glomerata* in morphological and cytogenetic characteristics. STEBBINS (1956a) reported that lower than normal frequencies of quadrivalents were produced experimentally in induced tetraploids of hybrids between the diploid *Dactylis* subspecies *lusitanica* and *judaica* by means of X-irradiation of the *judaica* pollen in the original cross.

In the present paper, cytological comparisons are made of natural tetraploids with colchicine-induced tetraploids from some of the recently-described diploid subspecies and hybrids. The new tetraploids have been compared with natural ones in their frequency of quadrivalents, trivalents and univalents at diakinesis and first metaphase and in the distribution of chromosomes at first anaphase. Evidence from chromosome cytology and from fertility has been sought for a possible advantage of inter-subspecific hybrid autotetraploids over pure ones in the natural origin of tetraploid *Dactylis*. Further, by comparing the quadrivalent frequencies in pure autotetraploids with those in inter-

subspecific hybrid ones, data have been sought which might be interpreted as preferential pairing, i.e. higher frequencies of quadrivalents in the pure than in the hybrids.

Preferential pairing has been interpreted by some (STEPHENS 1950) as evidence for small-scale structural differentiation of the chromosomes between two groups. MYERS and HILL (1943) found increases in mean quadrivalent frequencies of first inbred generation over parent from 3.00 to 3.66 and from 3.04 to 3.50 in two of eight inbred families, for which they proposed decreased preferential pairing conditioned by chromosome differentiation as a partial explanation. HANSON and HILL (1953) reported a mean quadrivalent frequency of  $1.75 \pm 0.10$  in 13 plants derived from crosses with a tetraploid having a reciprocal translocation, the presence of the translocation suggesting the presence of other, perhaps smaller, chromosome changes as well. In the material of tetraploid *lusitanica*  $\times$  *judaica* already mentioned (STEBBINS 1956a), the low mean quadrivalent frequency (1.52) of a "control" plant suggested to the author that "some structural differences exist already between the chromosomes of the Portuguese and Judean diploids".

Finally in the present paper, other analyses suggested by the data themselves are presented.

### B. Materials and Methods

The four groups of plants used in this study, listed with place of collection, are as follows: (1) Diploid subspecies *lusitanica* (*l*), near Sintra, Algueirão, Portugal; *ibizensis* (*i*), Ibiza, Balearic Islands, Spain; *juncinella* (*jn*), Sierra Nevada, near Residencia Universitaria, Spain; *smithii* (*sm*), Taganana, Tenerife Island, Canary Islands; *judaica* (*jd*), Judean Mts., Israel. (2) Diploid hybrids *i*  $\times$  *l*, *sm*  $\times$  *l*, *l*  $\times$  *jn*, *jd*  $\times$  *l*. (3) Natural tetraploids including typical *glomerata* (*g*), Golden Gate Park, San Francisco, California; *maritima* (*m*), near Sintra, Praia das Macãs, Portugal; collections of *hispanica* (*h*) from Alicante (301), Fraga-Candasnos (508), and Almeria (5457), Spain; Near-Eastern forms from Giresun (538) and Sivas (540), Turkey. (4) Tetraploid hybrids Golden Gate  $\times$  *maritima* (*g*  $\times$  *m*); and *4n* *ibizensis*  $\times$  *maritima* (*i*  $\times$  *m*).

Specimens of the diploids and natural tetraploids are being deposited in the University of California Herbarium, Berkeley, by Dr. G. L. STEBBINS. Further information on the sources of material may be found in MCCOLLUM (1958). The reader is referred to STEBBINS and ZOHARY (1958) for morphological, ecological and geographical descriptions of the subspecies. In the opinion of these authors, *ibizensis*, *juncinella* and *smithii* are extreme types in the pattern of morphological and ecological variation known for *Dactylis*, while subspecies *lusitanica* is an intermediate form having certain resemblances to *smithii*, *juncinella* and *aschersoniana*.

Diploids were treated with colchicine by flooding germinating seeds with 0.05, 0.1 or 0.2 percent solutions for two hours or for two successive two-hour periods. Subspecies *lusitanica* was treated March 3, 1955; *ibizensis*, *smithii*, *juncinella*, *i*  $\times$  *l*, *sm*  $\times$  *l*, and *l*  $\times$  *jn* were treated November 17, 1955. Rooted cuttings of *jd*  $\times$  *l* were soaked in 0.1 percent colchicine for nine hours August 26, 1956. Surviving plants, transplanted into flats of soil, were screened for tetraploidy by measuring the length of guard cells. For fertility studies, chromosome counts were made from each panicle on a chimeric plant, either in squash preparations of young anther wall tissue or at meiosis. Some cases were found of diploid and tetraploid sectors in the same panicle or even in the same anther. For studying pollen abortion, florets were collected in small envelopes on the morning of the day the anthers



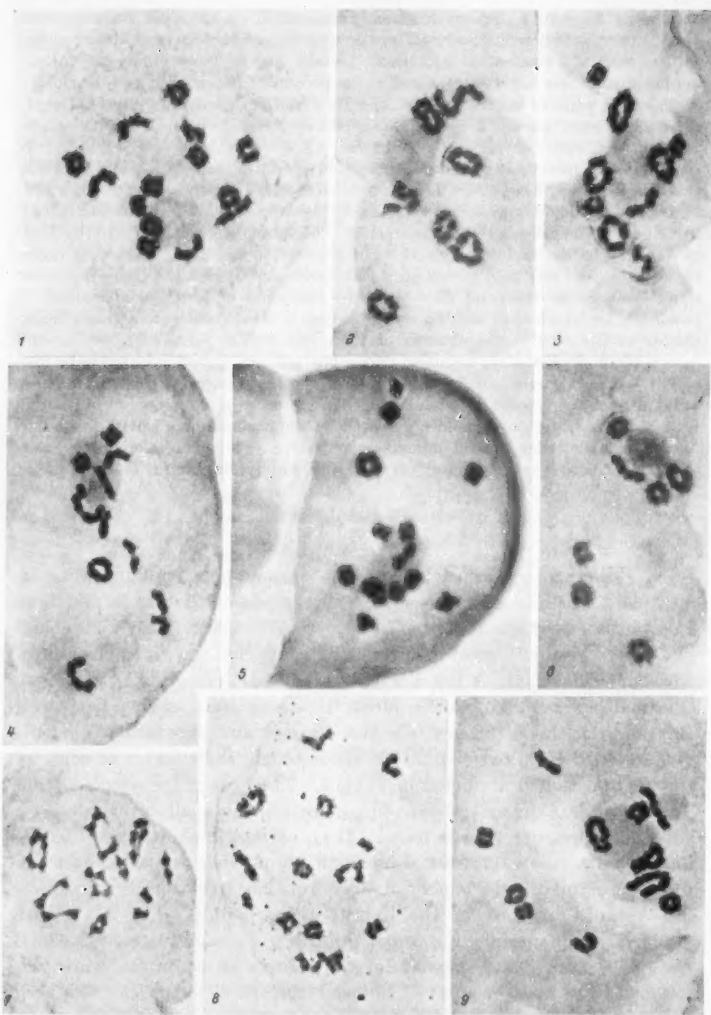
were ready to dehisce. Pollen abortion, as measured by improper staining with acetocarmine, was determined by microscopic examination of the contents of single anthers within a week after collection. Usually 200 or more grains per anther were counted. Seed set was measured as the percent of florets with well-developed caryopses in panicles harvested April and May, 1957. Usually 200 or more florets per panicle were examined. Both pollen and seed fertility values are for plants grown under winter greenhouse conditions.

Studies of meiosis in some plants were made during the spring and summer of 1956. However, most of the data on meiosis as well as on fertility were collected from plants brought into the greenhouse at Davis in the fall of 1956 and forced to flower by lengthening the photoperiod. Flowering continued from the first of January to the middle of March. Some material was also collected at other times from pot- and field-grown plants at Davis and Berkeley. These represent small fractions of the total data with the exception of tetraploid *lusitanica*  $\times$  *juncinella*, for which most information on meiosis is based on one plant grown in the garden at Berkeley in the summer of 1956, and diploid *juncinella*, one garden-grown plant at Berkeley, summer 1957. For meiosis, young anthers were dissected from the florets and fixed 24 hours in 1:3 glacial acetic acid and absolute ethyl alcohol, then stored in 70% ethyl alcohol. Studies of chromosome association were made from temporary slides prepared by the acetocarmine squash technique and examined under the oil immersion objective. The photomicrographs were made from both temporary and permanent slides by Mr. LORNE HARDAKER.

### C. Results

#### I. Quadrivalents

1. *Frequencies.* Tabulations of the chromosome configurations in tetraploid *Dactylis* microsporocytes were made at diakinesis and first metaphase (see Figs. 1—5 and 7—11, pp. 576, 593). Nearly all chiasmata were terminal, most quadrivalents being chains with three or rings with four chiasmata. A few Y's with three and "frying pans" with four chiasmata were also found. Most trivalents were chains with two chiasmata, although a few Y's and frying pans were also seen. Bivalents were rings or rods. Mean chromosome associations of some of the plants studied are listed in Table 1. These data are selected from Table 6 of McCOLLUM (1958), and do not include all of the data on which the present paper is based. If an asynaptic plant, *sm*  $\times$  *l* 638-4, and two extreme values based on small samples are omitted, the mean numbers of quadrivalents per cell range from 2.54 in subspecies *hispanica*, 301-Alicante, to 4.51 in the hybrid *4n i*  $\times$  *m* 6140. For a variance analysis of quadrivalent number (omitting *i*  $\times$  *m* 6140; *sm*  $\times$  *l* 638-1, aneuploid; and 638-4, asynaptic) the sources of variation were partitioned into four groups — induced tetraploid subspecies, induced tetraploid hybrids, natural tetraploid subspecies, and natural tetraploid hybrid (Table 2). Overall, the mean number of quadrivalents per cell was only slightly lower at metaphase than at diakinesis (3.44 vs. 3.50), so the samples of cells at the two stages were not separated in the analysis.



Figs. 1—9. Photomicrographs of meiosis. 1—3 Diakinesis in *maritima* 239-3 cells with 14 bivalents, 7 quadrivalents, and 4 quadrivalents + 6 bivalents. 4 Diakinesis in induced 4n *smithii* 635-13 cell with 3 quadrivalents + 8 bivalents. 5 Diakinesis in induced 4n *ibizensis* 634-8 cell with 1 quadrivalent + 12 bivalents. 6 Diakinesis in diploid  $i \times i$  cell with 7 bivalents. 7 First metaphase in induced 4n  $i \times jn$  639-6 cell with 5 quadrivalents + 4 bivalents. 8 Diakinesis in induced 4n *juncinella* 636-17 cell with 1 quadrivalent + 11 bivalents + 2 univalents. 9 Diakinesis in induced 4n *lusitanica* 585-9 cell with 3 quadrivalents + 8 bivalents (880  $\times$ )

Table 1. *Chromosome association in induced and natural tetraploids*

Sub-species or hybrid	Plant No.	Diak. or MI	Mean chiasmata per chromosome	Mean No. per cell				No. of cells
				Quad.	Tri.	Biv.	Univ.	
<i>l</i>	585-9	D	0.864	4.00	0.07	5.76	0.25	233
		M	.826	3.83	.27	5.60	.67	267
<i>i</i>	634-8	D	.894	3.89	.03	6.10	.17	71
		M	.878	3.41	.17	6.70	.46	54
<i>jn</i>	636-9	D	.812	3.63	.11	6.36	.44	113
		M	.822	3.82	.26	5.63	.68	128
	636-19	D	.778	3.37	.13	6.76	.60	68
		M	.800	3.14	.18	7.09	.73	125
<i>sm</i>	635-13	D	.754	3.43	.13	6.68	.52	499
		M	.756	3.47	.22	6.33	.79	190
<i>i</i> × <i>l</i>	637-10	D	.788	3.24	.21	6.74	.94	185
		M	.775	3.28	.33	6.29	1.29	226
	637-18	M	.798	3.15	.27	6.86	.86	194
<i>sm</i> × <i>l</i>	638-8	M	.784	4.24	.33	4.49	1.08	51
	638-1 <sup>a</sup>	D	.741	2.82	.58	6.20	1.65	106
	638-4	M	.716	2.88	.84	5.77	2.40	43
	638-4 <sup>b</sup>	M	.346	0.60	.57	5.59	12.73	126
<i>l</i> × <i>jn</i>	639-6	D	.881	4.30	.09	5.15	.24	229
		M	.855	4.00	.28	5.30	.57	47
<i>jd</i> × <i>l</i>	6106-6	D	.862	3.59	.05	6.65	.16	37
<i>g</i>	538-1	D	.824	2.83	.00	8.34	.00	110
	101-1	D	.947	3.91	.00	6.18	.00	88
	101-4	M	.906	4.09	.03	5.73	.06	65
<i>m</i>	239-3	D	.924	3.74	.005	6.51	.02	189
	239-5	D	.950	4.39	.02	5.16	.06	217
<i>h</i>	301-1	D	.779	2.54	.01	8.88	.04	151
	508-2	D	.901	2.95	.00	8.10	.01	169
		M	.887	2.93	.01	8.10	.06	123
	508-8	D	.750	2.56	.06	8.72	.14	251
	5457-8	D	.805	2.88	.04	8.16	.06	80
<i>g</i> × <i>m</i>	6137-2	M	.910	3.37	.01	7.18	.12	94
	6137-4	M	.898	3.51	.02	6.93	.05	148
	6137-5	M	.917	3.26	.02	7.41	.04	94
	6137-10	M	.884	3.43	.00	7.12	.04	89
<i>i</i> × <i>m</i>	6140-1	M	.835	3.36	.03	7.20	.09	101
	6140-2	D	0.915	4.51	0.00	4.95	0.05	113

a Aneuploid, 27 chromosomes.

b Asynaptic panicle.

Ordinarily the effect of variation in date of fixation should not be ignored. A number of studies have shown that chromosome pairing and chiasma frequency — which is correlated with quadrivalent frequency — may be changed to some extent by manipulating the environment in which the plants are grown, e.g. temperature, moisture,

Table 2. *Quadrivalents per cell*  
Strain and group means

Induced tetraploids				Natural tetraploids			
Subspecies		Hybrids		Subspecies		Hybrids	
<i>l</i>	3.88	<i>i</i> × <i>l</i>	3.22	<i>g</i> -101	3.99	<i>g</i> × <i>m</i>	3.42
<i>i</i>	3.47	<i>sm</i> × <i>l</i>	3.86	<i>g</i> -538	2.83		
<i>jn</i>	3.49	<i>l</i> × <i>jn</i>	4.19	<i>g</i> -540	3.53		
<i>sm</i>	3.42			<i>m</i> -239	4.06		
				<i>h</i> -301	2.54		
				<i>h</i> -508	2.76		
				<i>h</i> -5457	2.66		
Group means	3.57		3.55		3.31		3.42
Induced versus natural	3.56					3.34	

## Analysis of variance

Source of variation	Degrees of freedom	Mean square	Variance ratio	D. F. in F test
Total cells . . . . .	5433			
Groups . . . . .	3	23.489	<1	3/11
Strains within groups . . . . .	11	89.802	5.61 <sup>++</sup>	11/25
Plants within strains . . . . .	25	16.007	9.64 <sup>++</sup>	25/5394
Cells within plants . . . . .	5394	1.661		

<sup>++</sup> Significant at the 1% level of probability.

nutrition, etc. Various aspects of this topic are discussed by BARBER (1942), ELLIOTT (1955), GAUL (1953), GRANT (1952), HARTE (1953, 1954), HOFFMAN (1954), and LINNERT (1952, 1953). Few environmental studies of chromosome pairing involve quadrivalent frequencies in tetraploids. At high temperature (36° C, 24 hrs.) as compared to normal temperature, PAO and LI (1948) found an increased number of univalents and a decreased number of bivalents in both diploid and autotetraploid rye (*Secale cereale*) and a decreased number of quadrivalents in the tetraploid. VON BERG (1936) states that chiasma frequency in natural autotetraploid *Hordeum bulbosum* was lower in fixations of plants grown during conditions of low temperature including some frost (−2.9° C) than in fixations made under more favorable conditions. In spite of this asynapsis, the frequency of quadrivalents relative to trivalents, univalents, and bivalents was reported to be the same under both conditions.

In connection with the present study, five tetraploid plants of *Dactylis* were exposed to low temperatures (25–36° F) for four or five days during flowering in an attempt to modify the frequency of quadrivalents at meiosis. The procedure and results are described in MCCOLLUM (1958). Although in a few instances the frequency of chiasmata and quadrivalents was lower and the frequency of univalents higher

in the treated than in the control material, the effect of the cold was not consistent in all plants, nor even evident in some, suggesting that moderate changes in temperature played a relatively minor role in variation in quadrivalent frequency. Although MYERS (1943) found that the difference due to "years" in quadrivalents was not statistically significant in the six clones of *Dactylis* studied by him in two different years, MYERS and HILL (1942) obtained a significant difference between duplicate collections of twelve plants made two days apart in February. In the present study, "plants" mean square in the variance analysis is not independent of fixation date. However, as more than one plant is frequently represented on the same day of fixation, and some plants are represented by fixations on two different days a "fixation" sum of squares was partitioned in such a way as to provide a crude estimate of the relative importance of "plants" and "date of fixation" in causing variation in quadrivalent frequency. The effect of date of fixation was found to be sufficiently lower than the effect of plants as to be disregarded in the main analysis of quadrivalent frequencies (MCCOLLUM 1958). GRUN (1951) similarly found that variation in plants was much more important than variation in dates of fixation in affecting the quadrivalent frequency in alfalfa.

The variance analysis of quadrivalent frequencies (Table 2) showed that the differences between plants within strains and between strains within groups were highly significant, but that the differences between "groups" were not. The means listed in Table 2 (calculated as  $Sf X/N$ ) represent some of the comparisons made in the analysis. Taking these at face value, the hybrid induced tetraploids have as high a frequency of quadrivalents as the non-hybrid induced tetraploids. As a group, the induced tetraploids have a slightly but not significantly higher frequency of quadrivalents than the natural tetraploids. Among the latter, the four plants representing three different collections of *hispanica* had lower mean numbers of quadrivalents than are usually reported for natural tetraploid *Dactylis*, i.e. less than 3.0 per cell. It cannot be said certainly that such low values are characteristic of all plants in these collections nor of *hispanica* types in general, since ZOHARY (1955) reported a mean value of 3.28 quadrivalents per cell in a *hispanica* from Malaga, Spain. Nevertheless, the possibility of subspecies or locality differences in quadrivalent frequency as evidence of progress toward diploidization should be considered in any detailed cytological study of relationships among the natural tetraploids.

2. *Orientation*. If it is true that the disjunction of chromosomes from a quadrivalent is nearly always equal (2-2) when orientation of the quadrivalent is alternate (zig-zag ring or chain) whereas it is frequently unequal (3-1) if orientation is adjacent (GARBER 1954, 1955;

HILPERT 1957; THOMPSON 1956), the desirability of maximum zig-zag orientation and thus maximum regularity of chromosome distribution is obvious. The random probability of alternate versus adjacent orientation of the quadrivalents on the spindle has been considered to be one-half (GARBER 1955). A frequency of zig-zags higher than this would indicate non-random or "directed" orientation presumably under genetic control. THOMPSON (1956) has shown that heritable differences in type of disjunction from interchange configurations occur in rye, and GARBER (1954) found in tetraploid species of *Sorghum* ( $x=5$ )

Table 3. Frequencies of zig-zag quadrivalents at first metaphase

4n Sub-species or hybrid	Plant No.	Zig-zag quad. (%)	Total quad.	Total cells
<i>l</i>	585-9	38.9	684	170
<i>i</i>	634-8	30.4	184	54
<i>jn</i>	636-9	43.8	416	107
<i>jm</i>	636-19	45.7	392	125
<i>sm</i>	635-13	41.0	407	125
<i>i</i> × <i>l</i>	637-10	32.6	343	107
<i>i</i> × <i>l</i>	637-18	42.1	432	142
<i>sm</i> × <i>l</i>	638-8	41.2	216	51
<i>l</i> × <i>jm</i>	639-6	48.8	164	40
<i>g</i>	101-4	59.0	256	62
<i>m</i>	239-4	34.2	269	67
<i>h</i>	508-2	50.5	309	103
<i>g</i> × <i>m</i>	6137-4	62.8	519	148
<i>g</i> × <i>m</i>	6137-5	62.5	307	94
<i>i</i> × <i>m</i>	6140-1	47.5	339	101

that zig-zag quadrivalents predominate in *Sorghum australiense* and *S. plumosum* (87 to 97% zig-zags) but not in *S. leiocladum* (49–61%). High frequencies of zig-zags in other natural autotetraploids include 94% in *Hordeum bulbosum* (VON BERG 1936), 74 and 85% in *Arrhenatherum elatius* and *Agropyron cristatum* (MYERS and HILL 1940), 84 to 97% in *Agrostis canina* ssp. *montana* (JONES 1956a), and 70% in *Dactylis glomerata* (MÜNTZING 1933), all with  $x=7$ .

If zig-zag orientation is advantageous to the fertility

of an autotetraploid plant, one might expect natural selection to have favored genotypes which condition a maximum frequency of this type of orientation. According to this argument, natural tetraploids should have a higher frequency of zig-zags than do first-generation colchicine-induced tetraploids which have not been subjected to selection. In the *Dactylis* material studied here, in which the frequencies of zig-zags among quadrivalents at first metaphase were determined from samples with reasonably large numbers of cells (Table 3), this expectation was in general realized. However, all the plants fell below the 70% zig-zags reported by MÜNTZING, and only *g* 101 and *g* × *m* 6137 had frequencies above 50%. Presumably all of the induced, and possibly the natural tetraploids as well, have a non-directed orientation of quadrivalents.

3. *Binominal distribution.* Attempts to determine whether the probability of quadrivalent formation is the same for each of the seven sets of four homologous chromosomes in tetraploid *Dactylis* are per-



tinent to *Dactylis* cytogenetics in the following ways. If the chromosomes of the diploid subspecies are structurally differentiated, it is possible that this differentiation varies in degree from one set of four homologous chromosomes to the next. This variation could be made known by observing that different quadrivalent frequencies between sets occur in the induced tetraploids of subspecies hybrids but not in the induced tetraploids of pure subspecies, even though the overall mean quadrivalent frequency were the same in both types. Furthermore, HANSON and CARNAHAN (1956) have already suggested, on evidence that inbreeding depression was nearly as great in cultivated tetraploid orchard grass as is expected in a diploid species, that this form of *Dactylis* might be a segmental allopolyploid and exhibit both tetrasomic and disomic inheritance. STEBBINS (1950, pp. 325–326) discusses a stable type of segmental allopolyploid in which some sets of four chromosomes might be structurally homologous and able to form quadrivalents while other sets are so strongly differentiated as to form only bivalents.

Other possible causes of differences between non-homologous sets in frequency of quadrivalent formation should be eliminated before placing the responsibility on different degrees of structural homology leading to more or less preferential pairing among the various sets. For example, differences in chromosome length related to chiasma frequency may be important (MATHER 1937; REES 1957). TANAKA (1940) recorded significantly higher frequencies of quadrivalents among the long and medium length chromosomes than among the short ones in tetraploid *Carex siderosticta* ( $x=6$ ). Position of the centromere and number of "pairing blocks" (DARLINGTON and MATHER 1934; cf. OKSALA 1952) may also influence quadrivalent frequency. All the chromosomes of *Dactylis* are approximately the same length, have median or sub-median centromeres (LEVAN 1930) and usually have one chiasma per bivalent-arm, suggesting that there is one "pairing block" per chromosome arm in each of the seven chromosomes. Direct observation of differences in quadrivalent frequency between non-homologous sets is not possible in *Dactylis*, because individual differences in chromosome morphology have not been recognized at diakinesis and first metaphase in this species. However, if the probabilities of quadrivalent formation are the same for each set of four chromosomes and for every cell in an anther, the numbers of cells with 0, 1, 2, . . . 7 quadrivalents are expected to follow a binomial distribution; if they are not the same, deviation from the binomial may be expected. Agreement, of course, does not constitute proof.

The application of the binomial distribution to problems of chromosome pairing has been explained recently by HALL (1955) who used

the method on data collected from the literature to show that pairing frequency in hybrids between closely related species is uniform from one set of homologous chromosomes to the next but in hybrids of distantly related species it is non-uniform. POVILAITIS and BOYES (1956) found that the distribution of cells with varying numbers of quadrivalents in autotetraploid *Trifolium pratense* ( $x=7$ ) did not fit the expected binomial. Their sample of 383 cells, however, was made up by pooling smaller samples from 14 separate plants, some of which had slightly differing quadrivalent frequencies. MASIMA (1947b) states that the frequency distribution of cells with varying numbers of quadrivalents in autotetraploid *Linum angustifolium* ( $x=15$ ) corresponds to the binomial. Several other enumerations of quadrivalents found in the literature appear to be distributed in this way, e.g. JONES (1956b), but statistical verifications have not been included. In *Dactylis*, several reported distributions of quadrivalents are unimodal but apparently have not been tested against a theoretical binomial. In the present study, samples of at least 50 cells from each of 37 anthers were tested. Twelve of these distributions are shown in Table 4. To test the agreement of the observed distributions with theoretical binomial ones, both the variance ratio (F) and chi-square test were used. It is more convenient to obtain the statistics necessary for the F test than for the chi-square test, because it is not necessary to expand the binomial to compute its variance. Pertinent to the use and interpretation of the variance ratio in connection with binomial distributions is a discussion by YULE and KENDALL (1937, Ch. 19).

In the 37 samples tested, the conclusions from the variance ratios corresponded fairly closely with those from the chi-square tests. At the 5% level of significance, two distributions —  $g \times m$  6137-4 and  $4n$   $jn$  636-9 — did not agree with a binomial distribution according to the F test. Using chi-square, only one — 639-9 — did not agree. The deviating distributions could be expected by chance at the level of significance chosen. Furthermore, three sister plants of 6137, a second anther of 636-9, and two anthers of 636-19 did not deviate from the binomial. All distributions shown in Table 4 fit the binomial. From this evidence, there is strong reason to think that the probability of quadrivalent formation in all tetraploid *Dactylis* studied — induced and natural, subspecies and hybrid — is the same from one set of four chromosomes to the next within a cell and from one cell to the next within an anther.

4. *Correlation with chiasmata.* The variation in frequency of quadrivalents between plants and probably also between strains of *Dactylis* is associated in part with variation in frequency of chiasmata. MYERS (1943) calculated the correlation coefficient of quadrivalents and chias-

Table 4. Binomial distribution of quadrivalent numbers

Plant and stage	Distribution	Cells with number of quadrivalents:								Total cells (n)	Probability of quadriv. (p)	Variance*
		0	1	2	3	4	5	6	7			
<i>l</i> 585-9 meta.	E <sup>b</sup>	0.3	4.1	16.3	36.9	50.0	40.5	18.3	3.6	170.0	0.575	1.7106
	O <sup>c</sup>	1	3	11	46	49	40	15	5	170		1.6444
<i>jn</i> 636-19 meta.	E	2.0	11.1	27.0	36.5	29.6	14.4	3.9	0.5	125.0	0.448	1.7311
	O	2	10	25	41	29	15	3	0	125		1.5862
<i>sm</i> 635-13 diak.	E	1.4	8.6	22.9	33.9	30.0	15.9	4.7	0.6	118.0	0.470	1.7437
	O	1	11	19	38	29	12	7	1	118		1.8651
<i>i</i> × <i>l</i> 637-10 diak.	E	1.4	8.9	23.9	35.8	32.1	17.2	5.1	0.6	125.0	0.473	1.7449
	O	1	7	27	37	27	22	4	0	125		1.6357
<i>sm</i> × <i>l</i> 638-4 meta.	E	40.3	19.9	4.2	0.6					65.0	0.066	0.4315
	O	41	18	6	0					65		0.4399
<i>l</i> × <i>jn</i> 639-6 diak.	E	0.1	0.7	4.0	12.3	22.9	25.3	15.6	4.1	85.0	0.649	1.5946
	O	0	1	5	12	19	30	13	5	85		1.7036
<i>g</i> 101-1 diak.	E	0.2	2.1	7.9	16.5	20.5	15.3	6.4	1.1	70.0	0.555	1.7288
	O	0	3	7	16	21	17	4	2	70		1.7549
<i>m</i> 239-3	E	1.0	7.2	24.9	47.4	54.4	37.4	14.4	2.3	189.0	0.534	1.7419
	O	3	5	22	53	54	34	14	4	189		1.8445
<i>h</i> 301-1 diak.	E	6.5	25.8	43.9	41.5	23.6	8.0	1.5	0.2	151.0	0.362	1.6167
	O	2	32	47	34	26	9	1	0	151		1.5437
<i>h</i> 508-8 diak.	E	7.2	30.0	53.6	53.4	31.9	11.4	2.3	0.2	190.0	0.374	1.6389
	O	6	37	46	54	31	13	3	0	190		1.7498
<i>g</i> × <i>m</i> 6137-2 meta.	E	1.0	6.1	17.1	26.5	24.7	13.8	4.2	0.6	94.0	0.482	1.7477
	O	1	8	13	30	20	20	1	1	94		1.7846
<i>i</i> × <i>m</i> 6140-2 diak.	E	0.1	1.0	5.6	16.8	30.6	33.5	20.2	5.2	113.0	0.645	1.6028
	O	0	1	3	19	33	34	17	6	113		1.3007

a For the expected binomial, calculated as  $kpq$  where  $k=7$ ,  $q=1-p$ .b Expected distribution,  $n(p+q)^k$ .

c Observed distribution.

mata to be  $r=0.517$  (D.F. 7) in the nine clones of *Dactylis* he studied and  $r=0.462^{++}$  (D.F. 25) when the three replications per clone were used as paired values. In two inbred families containing six and twenty plants, a correlation coefficient,  $r=0.68^{++}$  was obtained (MYERS and HILL 1943). HANSON and HILL (1953) reported  $r=0.709^{++}$  for multivalents (mostly quadrivalents) and chiasmata in hexaploid *Dactylis*. In the present study, the mean numbers of quadrivalents and chiasmata per cell were paired in 43 plants (omitting *sm* × *l* 638-1 and 4) from which the correlation coefficient,  $r=0.539$ , and the regression coefficient of quadrivalents on chiasmata,  $b=0.164$ , were calculated. These values are significant at the 1% level of probability. By an analysis of covariance, MYERS (1943) showed that the differences between frequencies of quadrivalents in different plants were still significant after the means had been

adjusted for regression on chiasma frequency. Such an analysis is not included here, but it seems safe to conclude that no more than 30% ( $r^2$ ) of the variation in frequencies of quadrivalents among plants should be attributed to variation in chiasma frequency.

Differences between the quadrivalent frequencies of different plants are caused ultimately by differences in chromosome structure or in genetic constitution acting directly or indirectly on the frequency (1) of prophase association of all four homologs, (2) of change of pairing partners, and (3) of occurrence of chiasmata. Between-plant correlations of quadrivalents and chiasmata could result from causative elements common to both, for example genetic control of chromosome pairing or perhaps degree of chromosome homology. Structural differentiation of ichromosomes presumably will lower the frequency of quadrivalent pairing and promote bivalent pairing at early prophase and in addition decrease the frequency of chiasmata in those cases where "non-homologous pairing" did occur.

Within an individual plant or anther, however, the differences from cell to cell, ranging from 0 to 7 quadrivalents in tetraploid *Dactylis*, cannot be explained in terms of chromosome structure nor of genotype. Variability could be caused by differentiation of the internal environment, for example physiological gradients. It is more likely, however, that the explanation lies in the random occurrence or failure of occurrence of the several events necessary if one quadrivalent instead of two bivalents (or a trivalent and a univalent, etc.) is to be formed by a group of four homologous chromosomes. Randomness is suggested in the binomial distributions of cells described earlier. For discussions and literature reviews of several aspects of quadrivalent formation in autotetraploids, the reader is referred to ARMSTRONG (1954), DARLINGTON (1937), GILLES (1955), GOTTSCHALK (1954, 1955), GRUN (1951, 1952), LINNERT (1948, 1949), MASIMA (1947 a, b), MOFFETT (1936), MÜNTZING and PRAKKEN (1940), NORDENSKIÖLD (1945), OKSALA (1952) and UPCOTT (1939).

Cell-to-cell variation in chiasma frequency as one phenomenon associated with cell-to-cell variation in quadrivalent frequency was studied in *Dactylis* by MYERS (1943) who reported six highly significant within-plant correlation coefficients ranging from 0.559 to 0.304. Three others, 0.228, 0.126 and 0.113 were not significant, suggesting that variation in number of quadrivalents might be more closely associated with chiasma number in some plants than in others. In the present study, samples of cells from several anthers were large enough for the calculation of estimates of the within-plant relationship between quadrivalents and chiasmata. The possibility was considered that the dependence of quadrivalent formation on variations in frequency of chiasmata would be relatively less in the natural than in the induced

Table 5. *Intra-anther quadrivalent-chiasma regression and correlation*

Sub-species or hybrid	Plant No.	Diak. or MI	Total cells	Mean chiasmata per chrom. (x)	Mean quadriv. per cell (y)	Regression coeff. ( $b_{yx}$ )	Correlation coeff. (r)
l	585-9	D	68	0.871	4.18	0.091	0.123
	585-9	M	170	.835	4.02	.233 **	.334 **
	585-9	M	60	.795	3.55	.286 **	.441 **
jn	636-9	M	107	.827	3.89	.259 **	.424 **
	636-9	D	63	.800	3.38	.369 **	.635 **
	636-19	M	125	.800	3.14	.229 **	.316 **
	636-19	D	68	.778	3.37	.332 **	.457 **
sm	635-13	D	60	.773	3.83	.377 **	.512 **
	635-13	D	118	.735	3.29	.468 **	.657 **
i × l	637-10	D	125	.786	3.31	.220 **	.351 **
	637-10	M	107	.784	3.20	.311 **	.498 **
	637-10	M	61	.782	3.41	.316 **	.565 **
	637-18	M	76	.790	3.03	.302 **	.383 **
sm × l	638-4	M	65	.310	0.46	.065	.222
	638-4	M	61	.232	0.26	.077 **	.435 **
l × jn	639-6	D	85	.886	4.54	.329 **	.466 **
	639-6	D	66	.876	4.14	.423 **	.571 **
g	101-1	D	70	.946	3.88	.399 **	.376 **
	101-4	M	62	.906	4.11	.043	.051
g	538-1	D	61	.819	2.75	.350 **	.474 **
m	239-3	D	189	.924	3.74	.290 **	.318 **
	239-4	M	67	.926	4.01	.255 *	.268 *
	239-4	D	53	.912	4.02	.401 **	.478 **
	239-5	D	153	.953	4.59	.051	.053
	239-5	D	64	.942	3.92	.140	.143
h	301-1	D	151	.779	2.54	.363 **	.458 **
	508-2	D	62	.917	2.92	.364 **	.460 **
	508-2	M	103	.885	3.00	.328 **	.430 **
	508-8	D	61	.756	2.36	.420 **	.697 **
	508-8	D	190	.748	2.62	.405 **	.618 **
	5457-8	D	53	.792	2.79	.273 **	.413 **
g × m	6137-5	M	94	.917	3.26	.249 **	.306 **
	6137-2	M	94	.910	3.37	.181 *	.222 *
	6137-4	M	148	.898	3.51	.188 **	.248 **
	6137-10	M	89	.884	3.43	.172 *	.233 *
i × m	6140-2	D	113	.915	4.51	.271 **	.337 **
	6140-1	M	75	0.838	3.39	0.237 **	.348 **

\* Significant at the 5% level of probability.

\*\* Significant at the 1% level of probability.

tetraploids, the latter having in general lower chiasma frequencies and having had no opportunity for selection towards genetic control of quadrivalent frequency by means other than through chiasma frequency, e.g. prophase association and exchange of partners. No such difference between induced and natural tetraploids is evident (Table 5). High and

low correlation coefficients were obtained in both types. There are, however, significant differences at the 1% level of probability among the regression coefficients of the different anthers, as determined by an analysis of covariance using the method explained by SNEDECOR (1946, pp. 325—327). Also, when the correlation coefficients for the different samples of cells, at least within a single plant, are compared with their respective mean numbers of chiasmata per chromosome, there is some tendency for the correlation coefficients to be lower in anthers where chiasma frequency is relatively high than in anthers where chiasma frequency is lower. Obviously a quadrivalent requires a minimum of three chiasmata. Therefore if any cell has fewer than 21 total chiasmata, the maximum of 7 quadrivalents is by definition not possible in that cell. For this reason, a positive correlation is to be expected in any sample of cells, some cells of which have fewer than 21 chiasmata. The minimum number of chiasmata observed in any cell was 13 (with the exception of asynaptic *sm*  $\times$  *l* 638-4) and the maximum was 28. One might expect that the correlation would be greater in the range 13 to 21 chiasmata per cell than in the range 21 to 28, where chiasma frequency is high enough in every cell for the maximum of 7 quadrivalents to be formed (cf. LINNERT 1948). No single plant provided enough cells to test this idea, and the pooling of cells from plants of different genotypes, as this writer has done (MCCOLLUM 1958) possibly confounds factors responsible for between-plants correlations with those responsible for between-cells within-plants correlations. When a correlation diagram pooling 5427 cells was split into the range 13 to 21 chiasmata in which chiasma number is limiting and the range 21 to 28 chiasma in which it is not, the correlation coefficients obtained were 0.2976 and 0.2811 respectively, scarcely different from each other in absolute value, even though the difference is significant at the 1% level of probability. In any event, it seems obvious that in cells with fewer than 21 chiasmata, there is a direct causal relationship between chiasma and quadrivalent numbers in addition to any association of the two through a third element common to both. It is suspected that the correlation value for the complete range of 0 to 21 chiasma per cell (assuming the use of genetically homogeneous material having this wide range of chiasmata) would be much higher because of this relationship of "causation by definition" than the 0.2976 calculated from the smaller range 13 to 21 in a mixture of genotypes. Even in the range of 21 or more chiasmata, where there is no *necessary* cause and effect relationship, it may be argued that there is a correlation because an increase in number of chiasmata in a cell will decrease the probability that any set of four associated homologs will fall apart into two bivalents. Although the data presented here do little to clarify



the question, it seems reasonable to believe that the strength of the within-plant relationship of quadrivalent and chiasma frequencies may vary as individual plants vary in (1) general level of chiasma frequency, (2) general level of chromosome structural homology and genetic homozygosity, and (3) presence and kind of specific gene control in chromosome pairing behavior.

## II. Level of Chromosome Pairing

1. *Chiasma frequencies.* The formation of one quadrivalent rather than two bivalents by any set of four partially or completely homologous chromosomes is conditional on the occurrence of (1) at least one change of pairing partners during prophase association of the four homologs, and (2) chiasmata in sufficient numbers to connect each chromosome to one or more of the others. Change of partners at early prophase is difficult to study in most material. Chiasma frequency at diakinesis or first metaphase is more easily and routinely investigated.

To the extent that the number of chiasmata restricts the formation of quadrivalents, the study of chiasma frequency in the diploids provides background to any study of quadrivalent frequency in the induced tetraploids. At the diploid level, if a hybrid has fewer chiasmata or more univalents than its two parents, one possible interpretation is that the chromosomes of the parents are partially differentiated structurally and do not pair as well with each other as with their exact homologs (cf. STEPHENS 1950). Chromosome doubling of such a hybrid should increase the frequency of chiasmata. If poor pairing in the diploid hybrid has purely genetic causes, these may carry over to the tetraploid (BEASLEY and BROWN 1942). In either case — structural differentiation or upset in genetic control of chiasma frequency — bivalents are expected to be more frequent and quadrivalents less frequent in the hybrid tetraploid than in tetraploids of the two parents. In the first case, the cause is preferential pairing; in the second case, the cause is restriction on quadrivalents imposed by low chiasma frequency — two rod bivalents require only two chiasmata while one chain quadrivalent requires a minimum of three chiasmata.

MÜNTZING (1937) has reported an average of 0.776 chiasmata per chromosome in diploid *aschersoniana*. ZOHARY's studies (1955) indicate that hybrids between diploid subspecies of *Dactylis* have "normal" chromosome pairing and good fertility. This is true of the diploids studied here. Ring and rod bivalents with terminal chiasmata were the most common configurations at diakinesis and first metaphase. Very infrequent cross-shaped bivalents were also seen. The mean numbers of chiasmata per chromosome for the diploids are included in Table 6. There is no evident reduction in chiasma frequency in the

Table 6. *Chiasma frequency comparisons*

Subspecies or hybrid	Diak. or MI	Mean chiasmata per chromosome		Cells	
		Tetraploids	Diploids	4n	2n
<i>lusitanica</i>	D	0.870	0.930	320	128
	M	.832	.897	296	1349
<i>ibizensis</i>	D	.910	.930	115	954
	M	.863	.927	69	548
<i>juncinella</i>	D	.799	....	392	...
	M	.810	.686	272	176
<i>smithii</i>	D	.760	.728	589	688
	M	.756	.729	276	335
<i>i</i> × <i>l</i>	D	.782	.956	206	381
	M	.786	.876	427	830
<i>sm</i> × <i>l</i> <sup>a</sup>	D	.751	.918	122	221
	M	.759	.906	98	582
<i>l</i> × <i>jn</i>	D	.881	.899	231	284
	M	.831	0.878	76	357
<i>glomerata</i> -101	D	.947		88	
	M	.906		65	
<i>g</i> -538, 540	D	.834		129	
<i>maritima</i>	D	.933		499	
	M	.926		67	
<i>hispanica</i>	D	.803		651	
	M	.874		138	
<i>g</i> × <i>m</i>	D	.897		44	
	M	.902		425	
<i>i</i> × <i>m</i>	D	.915		113	
	M	0.835		101	
Diploid-tetraploid chimeras					
<i>l</i> 585-7	D	0.887	0.976	53	15
<i>l</i> 585-9	D	.864	.893	233	67
	M	.826	.888	267	1156
<i>i</i> 634-1	D	.910	.981	19	57
<i>i</i> 634-8	D	.894	.954	71	165
<i>sm</i> 635-13	D	.754	.722	499	444
	M	.756	.730	190	297
<i>sm</i> 635-40	D	.771	.762	37	100
<i>i</i> × <i>l</i> 637-18	M	0.798	0.837	194	225

<sup>a</sup> *sm* × *l* 638-4 omitted.

hybrids (see also Fig. 6). The values obtained in several plants of ssp. *smithii* were consistently lower than most of the others and are perhaps characteristic of this subspecies. The mean chiasma frequency of the only satisfactory fixation of *juncinella* (collected under different growing conditions from the others) was unusually low at 0.686 per chromosome.

In the tetraploids, as in the diploids, nearly all chiasmata are terminal at diakinesis and first metaphase. The mean numbers per chromosome are listed in Table 6 from which two generalizations may be made: (1) as a group the hybrid induced tetraploids have the same chiasma frequency as the tetraploids from pure subspecies, and (2) the natural tetraploids have a slightly higher chiasma frequency than the induced tetraploids. Among the natural ones, the *hispanica* types — especially Alicante 301 — are the lowest. The mean value 0.823 chiasmata per chromosome in a natural tetraploid was reported by MÜNTZING (1937), and among 9 clones studied by MYERS (1943), means ranged from 0.845 to 0.950. MYERS and HILL (1943) found one plant in a first inbred generation which had an average of 0.650 and two others with 0.760 and 0.795 chiasmata, while the remaining 23 inbred plants had averages above 0.800. No chiasma frequencies were published for the induced tetraploids studied by MYERS, i.e.  $4n$  *aschersoniana* and  $4n$  *aschersoniana*  $\times$  *voronovii*.

In the cases where material was obtained from both diploid and tetraploid sectors of the same plant, the chiasma frequency of the induced tetraploid could be compared more directly with its diploid counterpart (Table 6). Chiasma frequencies in *lusitanica*, *ibizensis*, and *i*  $\times$  *l* were slightly lower in the tetraploids than in the diploids, but in *smithii*, where it is already low, chiasma frequency is higher in the tetraploid than in the diploid.

It has been well demonstrated in cereal rye that differences in chiasma frequency are under genetic control (see especially REES 1955a, b; REES and THOMPSON 1956). Furthermore, species within a genus may have characteristically different mean chiasma frequencies per chromosome, such as GARBER (1950) has found in *Sorghum*.

2. *Trivalent and univalent frequencies.* The frequency of trivalents and univalents at meiosis is characteristically low in natural tetraploid *Dactylis glomerata* (ZOHARY 1955). No reports of the frequencies of these meiotic configurations in induced tetraploids of *Dactylis* have been found in the literature, although MYERS (1948) stated that induced tetraploid *aschersoniana* resembled *D. glomerata* in cytological behavior. Table 1 (p. 577) includes some of the mean numbers per cell of trivalents and univalents in the tetraploids studied here. Strain and group means, changed to numbers per hundred cells, are listed in Table 7. The natural tetraploids have the expected low frequencies of these configurations, averaging slightly less than 2 trivalents and 6 univalents per 100 cells at metaphase. MÜNTZING (1937) found 4 trivalents and 8 univalents per 100 cells in a plant of Skandia II. MYERS (1943) reported 0 to 9 trivalents per 100 cells in the 9 clones he studied; from 3 to 29 percent of the cells had at least one univalent (numbers not given).

MYERS and HILL (1942) found 0 to 27.3 univalents per 100 cells at metaphase among 20 open-pollinated plants, but they selected for analysis cells with no trivalents. Inbreeding for one generation increased the frequency of univalents two or three times in all families (MYERS and HILL 1943), but even some of their non-inbred material had a much higher frequency of univalents than the natural tetraploids

studied by me. However, the enormously higher frequency of trivalents and univalents in most of the induced tetraploids than in the natural ones with which they were compared (Table 7) is probably a real difference. Six to 15 times more trivalents and 8 to 15 times more univalents were found in the induced than in the natural tetraploids. The differences were great enough that no statistical tests were needed. Among the induced tetraploids, two of the hybrid types had slightly more trivalents and univalents than did the non-hybrids.

The group differences

Table 7. Mean trivalents and univalents per 100 cells

4n Sub-species or hybrid	Strain No.	Trivalents		Univalents	
		Diak.	Meta.	Diak.	Meta.
<i>l</i>	585	6.88	26.87	20.62	62.92
<i>i</i>	634	4.35	15.94	27.83	56.52
<i>jn</i>	636	11.99	19.12	39.54	69.48
<i>sm</i>	635	12.44	24.63	49.74	93.38
<i>i</i> × <i>l</i>	637	23.44	29.95	112.44	109.20
<i>sm</i> × <i>l</i>	638 <sup>a</sup>	38.46	40.28	100.00	126.39
<i>l</i> × <i>jn</i>	639 <sup>b</sup>	9.25	33.87	24.23	88.71
<i>g</i>	538,540	0.00	....	0.00	....
<i>g</i>	101	0.00	3.08	0.00	6.15
<i>m</i>	239	1.00	0.00	3.41	0.00
<i>h</i>	301	1.32	....	3.97	....
<i>h</i>	508	3.57	0.81	8.81	5.69
<i>h</i>	5457	3.75	13.33	6.25	13.33
<i>g</i> × <i>m</i>	6137	0.00	1.41	4.54	6.12
<i>i</i> × <i>m</i>	6140	0.00	2.97	5.31	8.91
Total induced ssp.		10.40	23.04	38.54	73.54
Total induced hybrids		16.70	31.72	67.48	109.14
Total natural ssp. and hybrids <sup>c</sup>		1.77	1.58	4.75	5.61

a 638-1 and 4 omitted.

b Includes some cells with 27 chromosomes.

c 6140 omitted.

between hybrids and non-hybrids were tested by variance analysis and found to be statistically significant at metaphase but not at diakinesis (McCOLLUM 1958). The different results for the two stages were perhaps caused by peculiarities of the data, e.g. some plants not represented in both analyses, unequal sample sizes, and differences between dates of fixation not controlled here.

3. *Chromosome distribution at first anaphase.* Previous work in natural tetraploid *Dactylis glomerata* has shown that chromosome distribution to the daughter nuclei at first anaphase of meiosis is equal (14-14) in most cells and gives rise to a high proportion of euploid gametes. Lagging and dividing univalents are not common. MÜNTZING (1937) scored the chromosomes in 8 out of 65 cells in plants of Skandia II and

Altai as separating 13-15, 13-1-14 or 13-2-13, a total of 12.3% irregular divisions; five and four cells per 100 in the two plants had one or two dividing univalents. MYERS (1943) reported 13 cells among 520 (2.5%) in which chromosomes were distributed 13-15, the rest 14-14. MYERS and HILL (1942) found significant differences among plants in percent of first anaphase cells with laggards, ranging from 1.7 to 34.0%. Laggards at anaphase were correlated with univalents at metaphase in 20 open-pollinated plants ( $r=0.72^*$ ) and in 7 inbred families ( $r=0.95^{**}$ ) according to MYERS and HILL (1942, 1943). In view of this correlation and the great differences between induced and natural tetraploids in univalent frequencies at metaphase, it is not surprising that great differences were found also in frequency of unequal chromosome distribution and of lagging and dividing univalents at anaphase (Table 8). There were 3.6% abnormal divisions in the natural tetraploids compared with 52.6% in the induced ones.

Table 8. Distribution of chromosomes at first anaphase

Sub-species or hybrid	Plant No.	Cells with distribution				Dividing laggards (1-6-Cell)	Irreg. cells (%)	Total cells
		14-14	13-15	12-16	11-17			
<i>l</i>	585-9	46	25	2	2	3	41.0	78
<i>jn</i>	636-17	33	11	4		16	48.4	64
<i>sm</i>	635-13	24	4	..		14	42.9	42
<i>i</i> × <i>l</i>	637-10	35	24	3		34	63.6	96
<i>sm</i> × <i>l</i>	638-3, 8	20	31	7		8	69.7	66
<i>l</i> × <i>jn</i>	639-6	49	32	6		4	46.2	91
<i>g</i>	101-4	139	7			2	6.1	148
<i>m</i>	239-5	104	1			1	1.9	106
<i>h</i>	301-1	49	..			..	0.0	49
<i>h</i>	508-2	96	1			1	2.0	98
<i>h</i>	5457-8	29	..			..	0.0	29
<i>g</i> × <i>m</i>	6137-2	97	5			1	5.8	103
Total 6 induced		207	127	22	2	79	52.6	437
Total 6 natural		514	14	..	..	5	3.6	533

4. *Cytological abnormalities.* The anthers in some panicles of the induced tetraploid *sm* × *l* 638-4 contained cells with large numbers of unpaired chromosomes at diakinesis and metaphase of meiosis — as many as 26 univalents in some cells. Samples of cells from other panicles of the same plant had nearly normal chromosome pairing. At diakinesis — the earliest stage studied — many univalents, some bivalents, and rarely, quadrivalents were observed (Fig. 13). At metaphase the univalents are scattered throughout the cell (Fig. 14); later most of them become oriented at the equatorial plate and divide equationally at first anaphase after the bivalents have separated. An 11-11 separa-

tion accompanied by the division of the remaining 6 univalents is shown in Fig. 15.

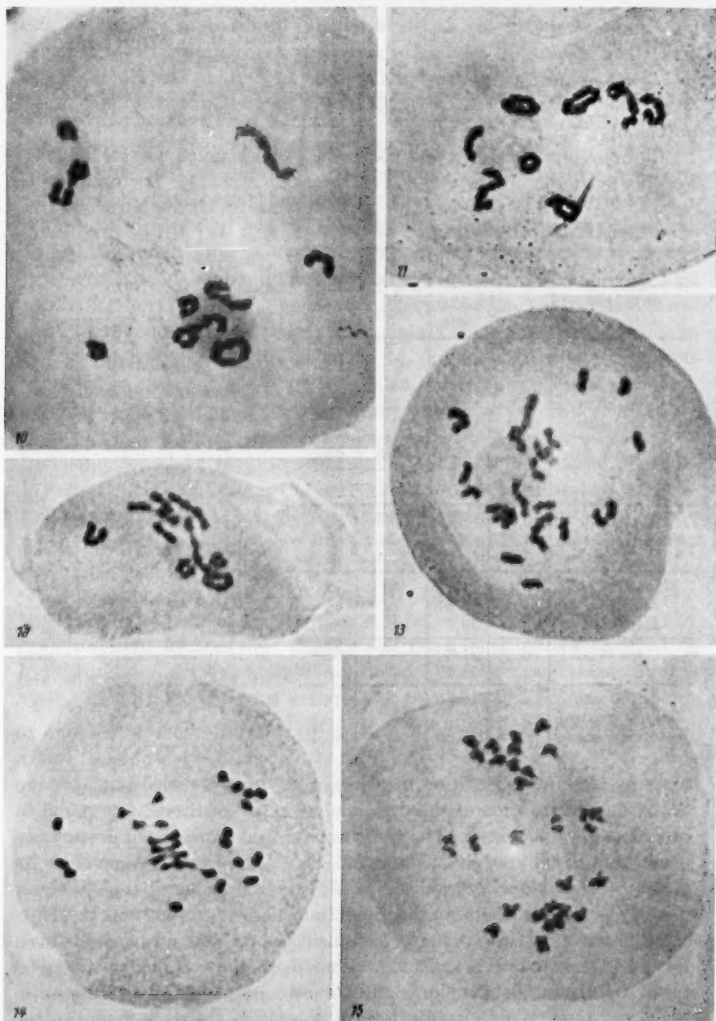
Extreme asynapsis is not typical of tetraploids from the hybrid  $sm \times l$ , because other plants of this combination, and actually other panicles of this same plant, were normal. One must consider the possibility that the asynapsis in this plant is merely an extension of the moderate pairing failure found in all the induced tetraploids. Recent tetraploidy, particularly combined with hybridity and its accompanying chromosomal unbalance, may have made chromosome association more susceptible to external influences on pairing and chiasma formation. As an alternative, perhaps the asynapsis-causing property of colchicine (cf. BARBER 1942; VAARAMA 1949) lasting over a substantial period of time (LEVAN 1939) is responsible. Or even a gene- or chromosome-mutation controlling chromosome pairing might have been produced by the colchicine in one sector of the plant, since colchicine appears to be mutagenic (HARPSTEAD, ROSS and FRANZKE 1954). The problem of asynapsis has recently been reviewed by GAUL (1954). Reference should also be made to papers of BROWN and MENZEL (1954) and PRAK-KEN (1943).

Chromosome irregularity of another kind, aneuploidy, also occurred in the first colchicine-induced generation tetraploids. Microsporocytes in tetraploid sectors of  $sm \times l$  638-1 had only 27 chromosomes (Fig. 12), while the diploid sectors had the expected 7 bivalents. A few microsporocytes of 638-4 also were interpreted as having 27 chromosomes, as were half the cells examined of  $l \times jn$  639-9.

### III. Fertility

Fertility data were obtained for all diploids and tetraploids grown in the winter greenhouse. Estimates of the mean percent of florets with well-developed caryopses and of normal-staining pollen are listed for each subspecies or hybrid in Table 9. The number of determinations for seed set averaged about 2.5 samples per plant and 3 plants per strain; for pollen, 5 samples per plant and 3 plants per strain. The induced tetraploids were compared with the diploids and with the natural tetraploids in two separate variance analyses. (For variance analysis tables, see MCCOLLUM 1958.) Each plant in one analysis was either diploid subspecies, induced tetraploid subspecies, diploid hybrid, or induced tetraploid hybrid. Each plant in the second analysis was either induced tetraploid subspecies, induced tetraploid hybrid, natural tetraploid subspecies, or natural tetraploid hybrid. In all cases but one, there were statistically significant differences, at the 5 or the 1 % level of probability, between plants within strains and between strains within groups. A significant variance ratio for groups was obtained





Figs. 10—15. Photomicrographs of meiosis. 10 Diakinesis in induced  $4n$   $l \times jn$  639-6 cell with 5 quadrivalents + 4 bivalents. 11 Diakinesis in induced  $4n$   $l \times l$  637-10 cell with 6 quadrivalents + 2 bivalents. 12 Diakinesis in induced  $4n$   $sm \times l$  638-1 aneuploid cell with 4 quadrivalents + 1 trivalent + 4 bivalents. 13—15 Asynaptic induced  $4n$   $sm \times l$  638-4 showing cells at diakinesis, first metaphase with 6 bivalents + 16 univalents, and first anaphase with an 11-11 distribution + 6 dividing laggards (880 $\times$ )

only for seed set in the diploid-tetraploid comparison, caused by the consistent decrease in seed set which occurred with chromosome doubling. That induced autotetraploids are less fertile than the diploids from which they were derived is the usual experience in studies of induced polyploidy.

Chromosome doubling lowered seed set as much in the diploid hybrids as it did in the diploid subspecies. Although the mean seed

set of all the natural tetraploids taken together (16.95%) was higher than that of the induced ones (8.77%), which might be expected in view of the lower frequencies of univalents and unequal chromosome distribution in the former, the difference was not statistically significant because only the *hispanica* types and the  $g \times m$  hybrid contribute to it. However, the poor seed fertility of the *maritima* and *glomerata* plants is not necessarily typical of these two strains and could have been caused by an unfavorable reaction to winter greenhouse condi-

Table 9. Seed set and pollen fertility

Subspecies or hybrid	Mean % seed set		Mean % good pollen	
	Diploid	Tetraploid	Diploid	Tetraploid
<i>lusitanica</i>	21.1	11.4	89	82
<i>ibizensis</i>	30.1	2.3	93	92
<i>smithii</i>	8.4	3.3	76	81
<i>juncinella</i>	19.9	10.0	89	91
$i \times l$	25.2	8.2	83	83
$sm \times l$	34.3	11.9	91	78
$l \times jn$	22.8	3.0	78	88
Mean 4 ssp.	19.37	8.01	88.3	83.3
Mean 3 hybrids	27.82	9.48	82.1	80.1
Mean 7 strains	24.09	8.77	85.3	82.2
<i>glomerata</i> -101		11.9		72
<i>maritima</i>		2.9		71
<i>hispanica</i>		39.2		89
$g \times m$		21.3		88
( $i \times m$ )		(8.4)		(85)
Mean 3 ssp.		16.30		78.2
Mean 4 natural tetraploids		16.95		80.4

tions or by low pollen concentration in the air when these plants were flowering. Even some of the diploids had poor fertility as compared to other fertility data obtained for these same strains under spring greenhouse conditions (STEBBINS and ZOHARY 1958; STEBBINS pers. comm.). As a group the diploid hybrids had a slightly but not statistically significantly higher seed set than the diploid subspecies (27.82 versus 19.37%); the difference between induced tetraploid hybrid and subspecies is even less (9.48 versus 8.01%). Reviews and discussions of pollen and seed fertility in induced tetraploids and the relation of fertility to the autopoloid versus allopoloid classification of polyploids are to be found in the works of ARMSTRONG (1950), DAVIS (1955), EIGSTI and DUSTIN (1955), MÜNTZING (1936, 1937), PARTHASARATHY (1953), POPE and LOVE (1952), RAMANUJAM and PARTHASARATHY (1953), SEARS (1941), STEBBINS (1947, 1949, 1950, 1956a) and SWAMINATHAN and HOWARD (1953).

#### D. Discussion

##### *I. The Question of Structural Differentiation of the Chromosomes*

The association of four chromosomes as two bivalents rather than as one quadrivalent may be extremely sensitive to the presence of small structural differences between the sets of two (SKIRM 1942). One cytological criterion for assuming the presence of small-scale structural differentiation has been the occurrence of lower quadrivalent frequencies in hybrid tetraploids than in related pure autotetraploids (STEPHENS 1950). Although some workers, for example BELL and SACHS (1953), in their study of amphidiploids in the *Triticinae*, have not been able to confirm the view that multivalent formation in an induced tetraploid is a more critical index of homology than bivalent pairing in the undoubled hybrid, the criterion of quadrivalent frequencies has provided a basis for speculation in some instances. MYERS and HILL (1943) suggested that decreased chromosomal differentiation resulting from inbreeding could have contributed to the increase in quadrivalent frequency found by them in two inbred families of cultivated *Dactylis*. IYENGAR (1944) found fewer quadrivalents in induced tetraploids of the hybrid *Gossypium herbaceum*  $\times$  *G. arboreum* than in tetraploids of the parent species. SWAMINATHAN (SWAMINATHAN and HOWARD 1953) found the same to be true among induced tetraploids of species and hybrids of *Solanum*, as did OKA, HSHEH and HUANG (1954) in tetraploid rice varieties and hybrids. In *Dactylis*, comparisons of this type have not been reported up to now. Among the three possible comparisons of hybrid induced tetraploids with the corresponding non-hybrids in this study, no good evidence for preferential pairing was obtained, although  $4n$  *ibizensis*  $\times$  *lusitanica* had slightly fewer quadrivalents than tetraploids of its two parents.

When large structural changes are present, smaller cytologically-undetectable ones may also be suspected. HANSON and HILL (1953) reported that quadrivalent frequency was unusually low in offspring of a *glomerata* plant known to have a translocation. Similarly, of the five induced tetraploids from the diploid hybrid *lusitanica*  $\times$  *judaica* reported by STEBBINS (1956a) to have mean numbers of quadrivalents lower than normal (1.07 to 1.62 per cell), four were derived from diploid hybrids which were partially sterile and heterozygous for at least one reciprocal translocation produced by X-irradiation of the *judaica* pollen in the original cross. Because the fifth plant, derived from a diploid hybrid which was fertile and had normal bivalent pairing, also had a low quadrivalent frequency, the suggestion was made that there were already structural differences between *lusitanica* and *judaica*. This fifth plant, however, also arose from irradiated *judaica* pollen, so that

one cannot be certain that the low quadrivalent frequency of the tetraploid is independent of the effects of X-rays. In this connection a tetraploid plant, *jd*  $\times$  I 6106-6 (Table 1), of non-irradiated origin has since been studied in which the mean number of quadrivalents per cell was found to be 3.59.

One additional technique has been applied to this question of structural differentiation. It seems reasonable that by demonstrating the presence of intra-genome (inter-chromosome) differences in degree of chromosome differentiation between subspecies, the assumption of inter-genomic differentiation between subspecies is supported. Such a demonstration was sought in *Dactylis* by testing for different frequencies of quadrivalent formation (or preferential pairing) among the seven sets of four homologous chromosomes in the hybrid induced tetraploids. However, from the evidence that even in these tetraploids (as well as in the non-hybrids and natural ones) the frequencies of cells with the various possible numbers of quadrivalents are binomially distributed, there is reason to believe that the probability of quadrivalent formation is the same for each set of four chromosomes. Therefore, until contrary evidence is obtained, perhaps from cytological studies of tetraploids from diploid hybrids between the most extreme morphological and ecological types, differentiation between the diploid subspecies of *Dactylis* must continue to be viewed as based mainly on gene mutation rather than on changes in chromosome structure.

The presence of more univalents in two of the induced tetraploid hybrids than in the induced tetraploid subspecies could be interpreted as caused by small chromosome differences between the diploids. It is, however, also reasonably interpreted as evidence of disturbed cellular physiology caused by gene disharmonies present in the diploid hybrids and aggravated by chromosome doubling. The occurrence of univalents in amphidiploids from interspecific and intergeneric hybrids of *Triticum*, *Agropyron* and related genera (ASHMAN and BOYLE 1955; POPE and LOVE 1952; SEARS 1941) and in  $F_2$ 's of interspecific *Poa* hybrids (GRUN 1955) has been explained in terms of physiological upsets due to hybridity, disharmonious gene interaction, etc.

## II. Induced versus Natural Tetraploid Comparisons

The low fertility in new autopolyploids is a well-known but poorly understood phenomenon (EIGSTI and DUSTIN 1955). Possible causes which have been suggested include unequal disjunction from multivalents, other meiotic irregularities including lagging univalents, specific genetic control, and disturbance in a balanced system of gene interactions (see discussions by ARMSTRONG and ROBERTSON 1956; MÜNTZING 1936; RAMANUJAM and PARTHASARATHY 1953). The series

of studies by MYERS and HILL (1942, 1943) and MYERS (1943) convinced these workers that the presence of quadrivalents was not contributing significantly to variations in the fertility of natural *Dactylis glomerata*, but that unpaired chromosomes at first metaphase which lagged at anaphase were correlated with and contributed to lowered fertility even though they might not be the chief cause. It is possible, of course, that low fertility and unpaired chromosomes are two independent symptoms of genically unbalanced cellular physiology rather than of chromosomal non-homology.

The fertility data collected in the present work are difficult to interpret. The differences in pollen abortion are small and follow no obvious biologically significant pattern. The induced tetraploids have poorer seed set than the diploids. It was expected that they would also be less fertile than the natural tetraploids, because they have had no opportunity to be selected for fertility. If they are compared only with the most fertile natural tetraploids, namely *hispanica*, this is indeed the case. However, the low fertility of *glomerata* and *maritima* in the samples studied cannot be ignored unless it is known that the conditions causing this poor seed set are not the same as the ones causing poor seed set in the induced tetraploids.

Covariation statistics for fertility and the meiotic phenomena were not computed. One obvious association however, whether or not accidental, is that *hispanica* types, with the lowest mean numbers of quadrivalents (Tables 1 and 2), had the highest percent seed set (Table 9). But although all the natural tetraploids had consistently fewer univalents and more regular chromosome distribution than the induced tetraploids they were not consistently higher in fertility. MYERS and HILL (1943) reported negative correlations between univalents and fertility in inbred families of *D. glomerata*. It is possible that in the present study sub-optimum environmental conditions for pollination and seed development have prevented the expected meiotically-determined differences in fertility to be clearly expressed. Finally, since the hybrid induced tetraploids were as fertile as the non-hybrid ones, there is no reason on the basis of fertility to prefer one type over the other as a more likely progenitor of natural orchard grass.

The causes are not clear why the chiasma frequencies at diakinesis and first metaphase are generally lower and the trivalent and univalent frequencies higher in the induced than in the natural tetraploids. The three meiotic phenomena are of course related to each other and to the frequencies of lagging and dividing univalents at first anaphase and of unequal distribution of chromosomes to the poles: MYERS and HILL (1943) reported highly significant negative correlation coefficients of chiasmata with metaphase univalents ( $-0.83$ ) and with anaphase

univalents ( $-0.74$ ) in two inbred progenies. The present paper is the first report of the differences in pairing between induced and natural tetraploid *Dactylis*, although MYERS (1945) compared induced autotetraploid *Lolium perenne* ( $x=7$ ) with natural *D. glomerata*. In that case, the mean numbers of quadrivalents in *Lolium* (2.92 to 4.83 per cell) were similar to *Dactylis* and were correlated with chiasma frequency ( $r=0.517^+$ ). Most notable among the meiotic irregularities in this *Lolium* material was the unequal distribution of chromosomes at first anaphase: of 40 cells examined, only 52.5% were 14-14; the rest were 15-13 or 16-12. Although the frequency of trivalents was low, the frequency of cells with univalents varied in different clones from 17.3 to 51.8%, with commonly two or more univalents per cell; 17.3 to 95.2% of cells at first anaphase had laggards which usually divided equationally. MYERS ascribed the irregular distribution to unequal disjunction from the quadrivalents and suggested that the more complicated types of quadrivalents and less terminalization of chiasmata in *Lolium* than in *Dactylis* could be responsible for the greater irregularity in *Lolium*. In the present comparisons of induced and natural tetraploids within *Dactylis*, however, this explanation cannot be held, as there seem to be neither qualitative nor consistent quantitative differences between the two groups in the formation of quadrivalents. The poorer pairing in the induced tetraploids is possibly caused by the action of the colchicine (1) directly on the cellular physiology, (2) in producing chromosome or gene mutation or (3) in chromosome doubling *per se*, with resulting unbalance of a mechanical, physiological or genetic nature.

### III. Improvement of Meiotic Regularity in Autotetraploids

As discussed by MÜNTZING (1936) and ANDERSON and SAX (1936), the maintenance of the euploid chromosome number and thus of fertility in experimental autotetraploids and of natural tetraploid races will depend in part on (1) bivalent pairing, (2) regular chromosome distribution from quadrivalents because of complete terminalization and zig-zag orientation, or (3) elimination of gametes and zygotes with aberrant chromosome numbers. The irregularities in colchicine-induced *Dactylis* tetraploids have significance to the extent that aneuploid gametes are viable and lead to a high frequency of aneuploid offspring. This may be no serious handicap to the use of these plants in breeding programs, although MÜNTZING (1937) found aneuploids in general to be less fertile and vigorous than euploids. More serious is the considerable nuisance value of aneuploidy in other types of genetic study involving these new autotetraploids.



Some works reported in the literature indicate that chromosome behavior may be changed by selection. Possibly selection has occurred to lower the quadrivalent frequency in the *hispanica* plants studied. GILLES and RANDOLPH (1951) reported that the mean quadrivalent number in autotetraploid maize selected for vigor and fertility over a period of ten years decreased from 8.47 to 7.46 (maximum 10). POVLAITIS and BOYES (1956) found fewer first metaphase univalents and abnormal sporocytes at first anaphase and telophase in the sixth colchicine-generation ( $C_6$ ) of Merkur variety autotetraploid red clover than in the  $C_1$  and  $C_2$  generations of Dollard variety, and fewer of these irregularities in the  $C_2$  than in the  $C_1$  of Dollard. In tetraploid rye (*Secale cereale*), PLARRE (1954) claims to have improved fertility and meiotic regularity, i.e. more bivalents and fewer quadrivalents, in one generation of selection for "regular" meiosis. BREMER and BREMER-REINDERS (1954), selecting for fertility in tetraploid rye over a period of six generations from the  $C_1$ , observed an increase in equal first anaphase distribution of chromosomes — 50% 14-14 in  $C_3$ , 75% in  $C_6$ . The frequency of laggards in  $C_6$  was only a third of that in  $C_1$ . Similar improvement was observed in regularity of second metaphase and tetrad stage. HILPERT (1957) succeeded in increasing meiotic regularity, i.e. more bivalents, fewer ring quadrivalents, trivalents and univalents, in tetraploid rye by selecting for vigor and high seed set during three generations. One generation of selection for regular meiosis had only negligible effect, although the frequency of zig-zag quadrivalents may have been increased slightly. On the other hand MÜNTZING (1951) concluded from comparisons of several tetraploid varieties differing in number of years selected for fertility that this selection had little or no effect on chromosome pairing. Similarly MORRISON's (1956) comparison of the rye varieties Tetra Petkus 1951 and T. P. 1954 showed only a slightly lower frequency of quadrivalents and unequal first anaphase chromosome distribution in the latter.

To the extent that fertility in autotetraploids depends on regular meiosis and equal chromosome distribution rather than on specific genetic factors, one would prefer a maximum of bivalents and/or zig-zag quadrivalents from which disjunction is equal and a minimum of ring quadrivalents, trivalents and univalents (HILPERT 1957). In other words it is not necessary to have all bivalent pairing, which is abnormal for an autotetraploid (MORRISON 1956), if all the chromosomes not paired as bivalents occur as zig-zag quadrivalents. The suggestion of MORRISON that one utilize asynaptic diploids from which to make tetraploids with a low chiasma frequency, and thus all bivalent pairing, should be tested but does not offer promise of success in *Dactylis* for at least two reasons. First, quadrivalent formation would probably

not be eliminated entirely. Chromosomes in the asynaptic *Dactylis* hybrid  $sm \times l638-4$  still formed quadrivalents. In cotton, induced polyploids were just as asynaptic as the asynaptic  $F_2$ 's (of *Gossypium hirsutum*  $\times$  *barbadense*) from which they were produced, but in spite of only 46% of the chromosomes paired, quadrivalents and trivalents were present (BEASLEY and BROWN 1942). Secondly, if unpaired chromosomes are more important than quadrivalents in lowering fertility in *Dactylis*, as the work of MYERS and HILL indicates, then a reasonably high chiasma frequency is desirable. In view of the correlations found between chiasmata, quadrivalents and univalents, selection for fewer quadrivalents caused by low chiasma frequency would lead to asynapsis and a higher frequency of aneuploid gametes. MYERS (1943) suggested, therefore, that selection for meiotic regularity should be based on simultaneous selection for decreased quadrivalents and increased chiasma frequency.

As discussed previously, STEBBINS (1956a) has accomplished the reduction in quadrivalent pairing in tetraploids by structural alteration of the chromosomes of the diploids with X-rays. However, if gene transfers are being attempted, it may not be desirable to suppress quadrivalent formation by such means which reduce intergenomal pairing in the hybrid tetraploids (BEAMISH, COOPER and HOUHAS 1957).

### Summary

Chromosome association and fertility have been studied in four diploid subspecies, three diploid subspecies hybrids, the induced tetraploids of these seven diploids, and several naturally-occurring tetraploids of the *Dactylis glomerata* complex.

1. The seed set of the induced tetraploids was significantly lower than that of the diploids from which they were produced and of some but not all of the natural tetraploids with which they were compared.

2. The mean numbers of quadrivalents per cell at meiosis in all the natural and induced tetraploids were typical of autotetraploids and within the range previously reported for *Dactylis*. Although statistically significant differences were found between plants and between strains, the chromosomes of the hybrid induced tetraploids formed quadrivalents as frequently as did those of the non-hybrids. There was thus no evidence for preferential pairing and consequently no support for the hypothesis of structural differentiation of chromosomes between the diploid subspecies.

3. Neither the hypothesis of structural differentiation of the diploid genomes, nor the suggested segmental allotetraploid nature of the natural tetraploids was supported by the cytological evidence that in all types of tetraploids examined, the frequencies within anthers of

cells with 0, 1, 2, . . . 7 quadrivalents were distributed binomially, consistent with the assumption that the probability of quadrivalent formation was uniform over all of the seven sets of four homologous chromosomes in a plant. Only the lower than usual quadrivalent frequencies in the *hispanica* plants examined, if proved to be typical, suggest progress toward diploidization in one form of natural tetraploid *Dactylis*.

4. The induced tetraploids differed from the natural ones in having a slightly lower frequency of chiasmata and a much higher frequency of trivalents and univalents at diakinesis and first metaphase, which presumably would lead to a greater frequency of lagging univalents at first anaphase and to unequal distribution of chromosomes to the first telophase nuclei. Unequal anaphase distribution was found to be greater in the induced tetraploids than in natural ones. One probable consequence of this irregularity of importance to the further use of these experimental tetraploids is the frequent production of aneuploid gametes and offspring. It seems possible, however, that regularity of chromosome distribution may be improved by selecting for high fertility, high chiasma frequency and maximum formation of bivalents and/or zig-zag quadrivalents from which disjunction is equal.

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Dr. G. D. McCOLLUM,  
Branch Experiment Station,  
Parma, Idaho, USA



From the Departments of Pathology and Botany, University of Wisconsin,  
Madison, Wisconsin

**AUTORADIOGRAPHIC AND MICROSPPECTROPHOTOMETRIC  
STUDIES OF DNA SYNTHESIS IN EXCISED  
TOBACCO PITH TISSUE\***

By

**NIRMAL K. DAS\*\*, KLAUS PATAU\*\*, and FOLKE SKOOG\*\*\***

With 4 Figures in the Text

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**Introduction**

Previously published microspectrophotometric data have shown that treatments with kinetin alone, indoleacetic acid (IAA) alone, and especially with both substances together induce deoxyribonucleic acid (DNA) synthesis in excised tobacco pith tissue cultured *in vitro* (PATAU, DAS, and SKOOG 1957). The statistical significance of the effect of kinetin alone on DNA synthesis, however, was not very satisfactory ( $P=0.03$ ). A re-investigation has, therefore, been carried out. The autoradiographic method was chosen in order to compare in the same material the two most frequently used methods for the study of DNA synthesis in individual nuclei, namely autoradiography and microspectrophotometry of Feulgen dye. Because of their very different nature these methods provide a check on each other. MOSES and TAYLOR's (1955a and 1955b) studies gave good correspondence between DNA synthesis as revealed by Feulgen dye measurements and by  $P^{32}$  incorporation.

To obtain autoradiographs tritiated thymidine was used. Labeled thymidine is known to be utilized for DNA biosynthesis with practically no diversion of radioactivity to other compounds (see REICHARD and ESTBORN 1951; FRIEDKIN *et al.* 1956; LU and WINNICK 1954). Autoradiographs by means of tritiated thymidine have first been described by TAYLOR *et al.* (1957).

**Material and Methods**

For the description of the plant material and methods of excision of pith tissue see DAS, PATAU, and SKOOG (1956). In the present study the pieces of pith were cut from a region, ca. 20 cm long, of the stem starting about 10 cm from the apex.

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\*\* Department of Pathology.

\*\*\* Department of Botany.

The tissue blocks were picked at random and cultured in groups of at least six in Petri dishes. Modified White's nutrient solution was used as before with agar but also as a liquid medium. In the main experiments the medium was supplemented, before autoclaving, with tritiated thymidine<sup>1</sup> and, except for controls, with IAA (2.0 mg/l) or kinetin (0.5 mg/l) or with both. The quantity of thymidine had been computed to give activities of about 0.2  $\mu\text{C}/\text{ml}$  in liquid medium and 0.9  $\mu\text{C}/\text{ml}$  in agar.

In the case of liquid medium each Petri dish (10 cm) contained four layers of lens paper thoroughly wetted with 2 ml of solution which was replenished daily with 1 ml of the same solution. In the case of agar medium the amounts were 12 ml per 5 cm Petri dish and 24 ml per 10 cm dish. All cultures were kept in diffuse light at room temperature of about 24°C. Samples were collected over a period of six days and fixed in 1:3 acetic alcohol for one day.

Microtome sections, 50  $\mu$  thick, were used exclusively. All were stained by the Feulgen procedure described by PATAU *et al.* (1957), except that a different batch; of basic fuchsin was employed (National Aniline Division; dye content 93% certification No. NF 64). Two groups of slides were prepared from each tissue block; one for autoradiographs and the other for the determination of frequencies of mitoses and new cells (each cell counted by its nucleus) or for DNA measurements. In the present investigation nuclei were counted only in alternate sections. This permitted the scoring of cut nuclei without risk of listing the same nucleus twice.

Autoradiographs were obtained (after Feulgen staining of the sections) by the stripping film technique of DONIACH and PELC (1950)<sup>2</sup>. The films were exposed for seven days. The process of drying squashed the sections, softened by hydrolysis, sufficiently to bring virtually all nuclei very close to the film<sup>3</sup>. Under a 43 $\times$  objective the vast majority of nuclei could be identified without any doubt as positive or as negative. However, some definitely positive nuclei produced noticeably fewer silver grains than most nuclei with comparable Feulgen dye contents. It can be presumed that such nuclei were at a fairly early stage of DNA synthesis. Slides from tissue cultured without tritiated thymidine showed only the general background of silver grains.

DNA measurements by the microspectrophotometric two-wavelengths method were made in the manner described by PATAU *et al.* (1957), except that different wave lengths were chosen. The purpose was to obtain stronger absorption by the Feulgen dye and, thereby, to reduce errors caused by non-specific absorption. Starting form  $\lambda_2 = 5200 \text{ \AA}$ ,  $\lambda_1 = 4960 \text{ \AA}$  was determined (for the procedure see PATAU *et al.* 1957).

In the present study DNA measurements were made only in an experiment in which IAA and kinetin were not used. From each of 18 tissue blocks 20 interphase nuclei (in one case 19) were randomly selected, 10 of which were measured in a first, the remaining ones in a second "run", each run covering the whole experiment. The order in which these blocks were arranged in the first run was reversed in the second run as a routine precaution against systematic errors resulting from fading of the Feulgen dye.

To test for fading 29 nuclei were re-measured after about 11 weeks. For each nucleus the ratio,  $\gamma_1/\gamma_2$ , of the first by the second "replication" was computed. The

<sup>1</sup> The tritiated thymidine was purchased from Schwarz Lab. Inc.; specific activity: 118 mC/m.mole; stock solution: 220  $\mu\text{C}/\text{ml}$ ; radiochemical purity: 55%.

<sup>2</sup> The authors wish to thank Dr. W. PLAUT for help with this method.

<sup>3</sup> This does not hold true for slides which had been made permanent after the Feulgen staining.

mean of the 29 ratios,  $1.005 \pm 0.033$ , does not indicate any fading. In our previous measurements considerable fading had been observed which for an 11 week period amounted to a ratio of  $1.0322^{5/11} = 1.190$  (cf. PATAU *et al.* 1957). Since the latter value was derived, by interpolation, from the ratios  $\gamma_1/\gamma_2$  of 571 nuclei its standard error must be much smaller than that of 1.005. The difference (1.190—1.005) is, therefore, highly significant. It can doubtlessly be ascribed to difference in the two batches of basic fuchsin used, because the procedures for making the Feulgen solution and the Feulgen stained slides were the same and the slides were kept in the refrigerator in both studies.

In a previous investigation of the mitotic rate (DAS *et al.* 1956) considerable biological variation between tissue blocks had been found. In the present material a similar, highly significant, variation in the frequency of DNA synthesis was evident in the data from autoradiographs as well as from DNA measurements (Table 1). For tests of significance the frequency of radioactive nuclei per block

Table 1. *Biological variation in the incidence of DNA synthesis: autoradiographic and microspectrophotometric data*

Kinetin, 6 days			Control, 2 days					
			Without H <sup>3</sup> -thymidine			With H <sup>3</sup> -thymidine		
Number of scored nuclei per tissue block								
Radioactive		Total	DNA content		Total	DNA content		Total
+	—		<27.86 *	>27.86		<27.86 *	>27.86	
45	163	208	15	5	20	9	10	19
91	136	227	11	9	20	6	14	20
99	126	225	13	7	20	17	3	20
94	127	221	13	7	20	11	9	20
35	49	84	5	15	20	4	16	20
			8	12	20	12	8	20
364	601	965	65	55	120	59	60	119
$\chi^2_4 = 30.0$ ; P = $5 \times 10^{-6}$			$\chi^2_5 = 13.9$ $\chi^2_5 = 21.2$					
			$\chi^2_{10} = 35.1$ ; P = $10^{-4}$					

\*  $27.86 = 4 C \sqrt{2}$ .

was transformed into  $\arcsin \sqrt{\text{percentage}}$  to render mean and variance independent (SNEDECOR 1946, Table 16.8). The term "average frequency" of radioactive nuclei in several tissue blocks will henceforth refer to the percentage derived, by the inverse transformation, from the mean of the transformed values. In the case of DNA measurements the statistical tests were performed with the geometric mean of the DNA contents of 20 (19) nuclei per tissue block (cf. PATAU *et al.*, p. 958, 1957).

## Results

### A. Tests of methods

Sections from tissue blocks which had been treated with tritiated thymidine were digested with 0.5 mg/ml of deoxyribonuclease (dissolved in McIlvaine's buffer, pH 6.7, with 0.003 M of magnesium sulfate) for 4 to 6 hours at ca. 38° C. This abolished the Feulgen stainability and the

radioactivity of nuclei (both checked in control slides) which shows that in the fixed material the tritium was present only in the DNA. SAVAGE and PLAUT (in the press) made the same observation in onion root tips.

As stressed before, virtually all radioactive nuclei stand out strikingly in the autoradiographs while all other nuclei give no indication of being radioactive (Fig. 1 above). This suggests that the retained thymidine has been incorporated only during DNA synthesis. The absence of appreciable turnover of thymidine or exchange of hydrogen atoms was further borne out by the following experiment performed with tissue blocks not treated with IAA or kinetin. It will be shown below that in such "control" tissue many nuclei underwent DNA synthesis during the first two days but few, if any, thereafter. Sixteen tissue blocks were treated for two days with tritiated thymidine, six were then fixed while ten were transferred to medium containing 10 mg/l of unlabeled thymidine (a much higher concentration than that used for tritiated thymidine). Five blocks were fixed after two and five after two more days on this medium. The average frequencies of radioactive nuclei in the three samples were 20.6%, 21.5% and 23.5% respectively (transformed values:  $27.0 \pm 4.4$ ,  $27.6 \pm 2.7$ ,  $29.0 \pm 2.4$ ). This close agreement precludes any appreciable turnover or exchange in the present experiment.

A statistical test was undertaken to check whether the presence of agar in the medium would influence the frequency of DNA doubling. The

Table 2. *Test for an effect of agar in the culture medium on the frequency of radioactive nuclei.*

In each case the t-test was applied to the difference between two means of transformed values: (2 tissue blocks from agar) — (3 tissue blocks from liquid medium)

Treatment	Duration of treatment in days								
	1			2			6		
	$t_2 \rightarrow P \rightarrow \chi^2_2$			$t_2 \rightarrow P \rightarrow \chi^2_2$			$t_2 \rightarrow P \rightarrow \chi^2_2$		
Control .	+0.51	0.64	0.89	+0.36	0.74	0.60	+1.08	0.36	2.04
Kinetin .	-4.46	0.021	7.73	+0.89	0.44	1.65	+0.77	0.51	1.35
IAA . . .	-0.76	0.50	1.39	+0.19	0.86	0.30	+1.62	0.22	3.03
Kinetin and IAA	-3.29	0.047	6.12	+0.097	0.93	0.15	-1.73	0.18	3.41
$\chi^2_2$			16.1			2.7			9.8
P			0.04			0.95			0.27
$\chi^2_{16} = 12.5$ ; $P = 0.71$									

results shown in Table 2 do not indicate any such influence after two or six days of treatment. After one day the frequencies of radioactive nuclei seem to be lower in tissue cultivated on agar than in tissue from

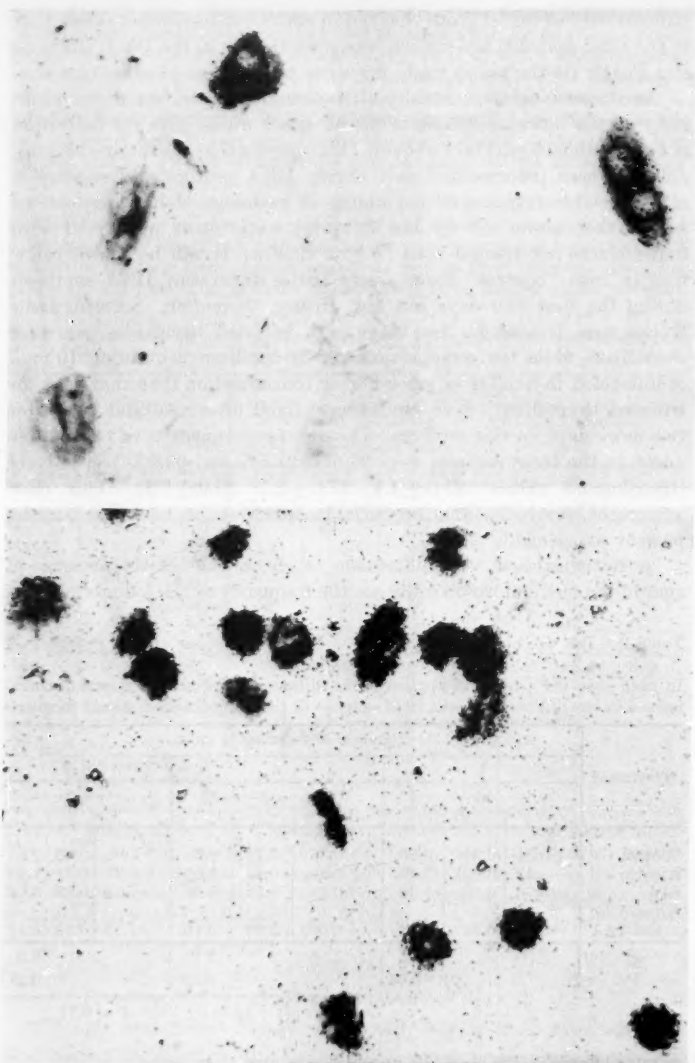


Fig. 1. Autoradiographs of Feulgen stained nuclei. Above: positive and negative nuclei (IAA, 2 days); below: patch of new cells — all positive (IAA + kinetin, 6 days; the high density of the background is exceptional).  $\times 515$

liquid medium<sup>1</sup>. This difference, if real, might reflect a decreased frequency of DNA synthesis or merely a slower penetration of tritiated thymidine from agar into the tissue.

The possibility that tritiated thymidine itself has an effect on the frequency of DNA doubling was tested by DNA measurements in 12 control tissue blocks of which six had been cultivated for two days on agar medium with and six on agar without tritiated thymidine. The means (each of six geometric mean DNA contents) were 27.9 and 26.1 respectively, with  $t_{10}=0.59$  and  $P=0.57$ . This does not indicate any thymidine effect (see Table 1 in which the difference between 65:55 and 59:60 is also insignificant).

### B. DNA synthesis in control tissue

It has been mentioned above that DNA synthesis occurred in many nuclei in cultures without added kinetin or IAA. This was revealed by autoradiographs in two independent experiments performed about two months apart and with material from different batches of plants. In one case about 21% of radioactive nuclei were found after two days, and 27% after two more days (average from six tissue blocks each). In the other case the frequencies were about 15% after two days, and 25% after four more days (five tissue blocks each; Fig. 2). An analysis of variance showed that the differences between the result of the two experiments are insignificant. The indicated increase in the frequency of radioactive nuclei after the first two days was not significant even in the pooled data from the eleven blocks of tissue each at two and at four and six days ( $t_{30}=1.65$ ;  $P=0.12$ ).

The occurrence of DNA synthesis within the first two days in control tissue was confirmed by photometric DNA determinations. Measure-

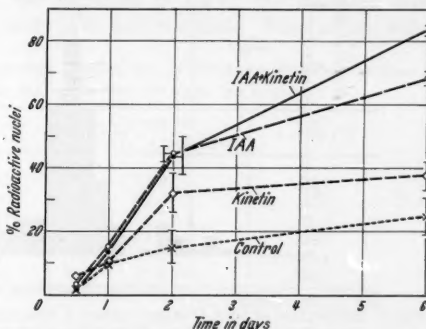


Fig. 2. Induction of DNA synthesis by 0.5 mg/l of kinetin, 2 mg/l of IAA, and both together. Ordinates correspond to means and standard error limits computed from transformed values (see p. 608). Number of tissue blocks per point: 3 at  $\frac{1}{2}$  and 1 day (except 2 for kinetin at  $\frac{1}{2}$  day), all cultured on liquid medium; 3 blocks grown on liquid medium and 2 grown on agar at 2 and 6 days (for justification of pooling see p. 612). Mean number of nuclei scored per block: 226

<sup>1</sup> For this reason frequencies obtained from blocks grown on agar for one day have not been included in Fig. 2.

ments of 20 (in one case 19) nuclei were made in each of six tissue blocks fixed on zero day, in six blocks after two days of culture without added tritiated thymidine, and six blocks cultured with tritiated thymidine (these last six tissue blocks were the same from which the above frequency of 21% of radioactive nuclei at two days had been obtained). As tritiated thymidine had no noticeable effect on the nuclear DNA content at two days (see above) the last two groups of values can

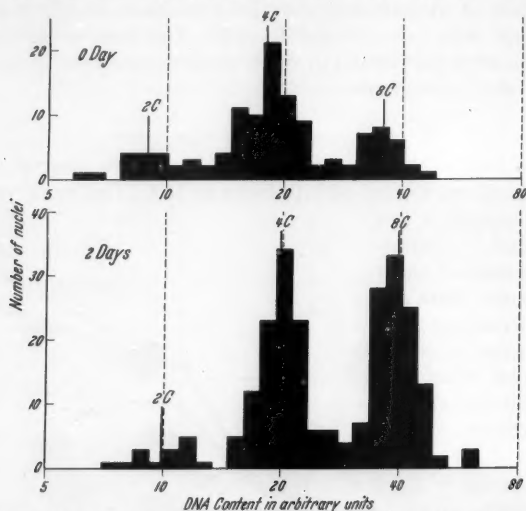


Fig. 3. Distribution of DNA (Feulgen) values of interphase nuclei (one "replication" each) in control tissue. Number of tissue blocks: 6 on 0 day and 12 at 2 days

be pooled. Fig. 3 shows three definite DNA classes which by comparison with our previously published results (PATAU *et al.* 1957) can be identified as 2C, 4C and 8C. Because only one replication per nucleus had been determined the considerable scattering of the measured values around the class values does not necessarily indicate the presence of nuclei with genuinely intermediate DNA contents. The best fitting class values (in arbitrary units) at zero and two days are given by  $C=4.51$  and  $C=4.94$  respectively. This is an apparent increase of the same magnitude as that observed in the previous study (PATAU *et al.* 1957). It was then shown that this increase cannot be considered as real, but rather as a systematic error caused by an increase with time in non-specific absorption in cultured tissue. Therefore, all DNA values from two day samples were corrected by the factor  $4.51/4.94=0.913$ . The mean of the geometric mean DNA contents (corrected) from 12 tissue blocks at two days was 5.5C, that of the six blocks on zero day



4.2C. The difference is significant ( $t_{16}=2.67$ ;  $P=0.017$ ), and would have been even more significant without the correction.

PATAU *et al.* (1957) gave a formula for computing the average number,  $s/n$ , of DNA doublings per nucleus from the geometric mean DNA contents at the beginning and at the end of a given period. The formula presupposes that no mitoses occur during that period. This condition was fulfilled since not a single mitosis has been observed in any control slide. Another condition, no DNA synthesis at the beginning and at the end of the period, was probably not strictly fulfilled but is quantitatively of no importance. In order to obtain an estimate not only of  $s/n$  but also of its accuracy, the formula was applied to each of the logarithms of the six geometric mean DNA contents,  $\log \gamma_0$ , at zero day, together with the logarithm of the over-all geometric mean from all 12 tissue blocks at two days:  $\log \gamma_2 = 1.382$ . Since this procedure makes no allowance for the variation of  $\log \gamma_2$ , the standard error obtained in Table 3 most likely is an underestimate. Even so, the value

Table 3. *Computation of the average number,  $s/n$ , of DNA doublings per nucleus in control tissue during the first two days (see text)*

Block No. (0 day)	$\log \gamma_0$	$s/n$ in per cent $100 (\log \gamma_2^* - \log \gamma_0) / \log_2$	Arc sin $\sqrt{\%}$
1	1.280	33.9	35.6
2	1.190	63.8	53.0
3	1.368	4.7	12.5
4	1.250	43.8	41.4
5	1.240	47.2	43.4
6	1.329	17.6	24.8

Mean:  $35.1 \pm 5.9$

\*  $\log \gamma_2 = 1.382$ .

$35.1 \pm 5.9$  (5 d.f.) does not differ significantly from  $25.0 \pm 3.0$  (10 d.f.) the mean of eleven transformed percentages of radioactive nuclei at two days. The results from the two entirely different methods are, therefore, in satisfactory agreement. An over-all estimate of the frequency of nuclei which had undergone DNA doubling during the first two days in control tissue is obtained as follows. The mean of the two transformed values (35.1 and 25.0) weighted by the inverted variances is 27.07. This corresponds to a frequency of 20.7%.

### C. Effects of kinetin alone

Kinetin alone induced DNA synthesis during the first two days of treatment (Fig. 2). The slight increase in the frequency of radioactive nuclei during the next four days, while obviously not significant, parallels that in control tissue. For a statistical test the deviations of the transformed percentages from the common regression at two and at six

days were pooled for the kinetin treatment and for controls. With  $t_{17}=2.82$  and  $P=0.012$  the difference of about 15% between kinetin and control tissue is significant.

#### D. Effects of IAA alone and of IAA and kinetin together

The frequency of radioactive nuclei in tissue treated with IAA alone was considerably higher than that in tissue treated with only kinetin (Fig. 2). A similar difference was also indicated in the data of PATAU *et al.* (1957). It is noteworthy that with IAA, in contrast to kinetin, there was still a highly significant increase in the frequency of radioactive nuclei during the period from two to six days. It is, however, possible that DNA synthesis in the presence of added IAA but without added kinetin did not continue through the whole period.

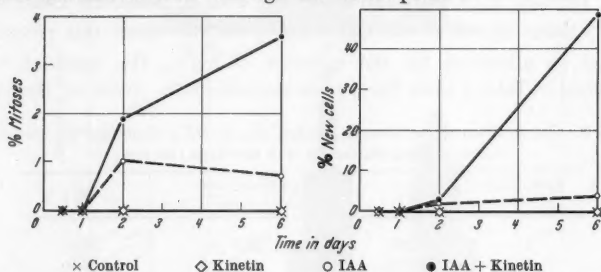


Fig. 4. Frequencies of mitoses and new cells as functions of the duration of treatments with IAA (2 mg/l) and/or kinetin (0.5 mg/l). Number of tissue blocks per point: 3 at  $\frac{1}{2}$  day, 5 at 1 through 6 days. Mean number of cells scored per block: 225

During the first two days the treatment with kinetin and IAA together had about the same effect on the frequency of radioactive nuclei as that with IAA alone. During the next four days, however, the combined treatment was much more effective than IAA alone (the difference between about 32% and 17% of non-radioactive nuclei is highly significant: Fig. 2) which also confirms previous findings (PATAU *et al.* 1957).

#### E. Mitosis and cytokinesis

The present observations (Fig. 4) on mitosis and cytokinesis, in terms of recognizable new cells, are in good general agreement with previously published results (DAS *et al.* 1956). Again, not a single mitosis was found in tissue not treated with IAA regardless of the presence or absence of kinetin and only the combined treatment with IAA and kinetin continued to induce cell division leading to patches of embryonic cells. The great majority of nuclei in such cells were radioactive as expected (Fig. 1 right); the exceptions, 17 out of 414, may have been "old" cells misclassified as "new".

### Discussion

The results from tritiated thymidine incorporation and from Feulgen dye measurements, new as well as previously published ones (PATAU *et al.* 1957), support each other well. Both methods, each with distinct advantages, are reliable tools for investigating DNA synthesis in individual nuclei.

The specific purpose of the present investigation was to test once more the effect of kinetin alone on DNA synthesis. The statistical significance ( $P_1 = 0.03$ ) of the previously observed effect was somewhat questionable (PATAU *et al.* 1957). The present results, with  $P_2 = 0.012$ , confirm that kinetin causes a substantial increase in the frequency of DNA synthesis, in the present case by about 15%. Since the above two P-values were the outcome of independent tests of the same null-hypothesis a total measure of significance can be obtained as follows (FISHER 1934): the two P-values (using for  $P_1$  the more accurate value 0.033) correspond to  $\chi^2_1 = 6.82$  and  $\chi^2_2 = 8.85$  respectively. These add up to  $\chi^2_4 = 15.67$  which yields  $P = 0.0035$ .

The present results are also in good agreement with the previous ones (PATAU *et al.* 1957) in regard to the stimulation of DNA doubling by IAA alone and by kinetin and IAA combined. Differences in conditions such as the season and other factors affecting the plant material may readily account for minor discrepancies between the previous and present results. However, in one respect the latter differ rather sharply from the former. In the earlier study no significant DNA doubling was found in control tissue cultivated in the absence of added kinetin or IAA, this time it occurred in about 20% of nuclei during the first two days.

PATAU *et al.* (1957) considered it likely that some DNA doubling took place in pith tissue *in situ* and probably also in culture. Therefore, it would not have been surprising to find a few radioactive nuclei in control tissue. The presently observed frequency of such nuclei is, however, of a magnitude that would have shown up in the DNA measurements of the previous study had it existed. It is conceivable, but unlikely, that in that study both tissue blocks available as zero day control happened to be exceptional ones. Their geometric mean DNA content was 5.2C as compared with 5.4C in eight control blocks fixed from one through 3½ days. In the present case the mean of six geometric mean DNA contents (each from a different block) on zero day was a mere  $4.2 \pm 0.26C$  as compared with 5.5C from 12 blocks fixed after two days. Most likely the frequency of nuclei in higher DNA classes in freshly excised tissue was generally higher in the old study than in the present one. This would indicate a difference in "physiological age" of the pith tissue which might account for the discrepancy. It should

also be mentioned that in the old experiments the tissue was cultured in darkness, but not in the present ones. Light has previously been shown to promote growth and differentiation in excised stem segments of tobacco (SCHMITZ 1951; STONE 1951), an effect which may be mediated through kinetin or kinetin-like substances in the tissue.

It appears almost certain that DNA synthesis in about 20% of nuclei during two days in control cultures greatly exceeds the normal rate *in situ*, because with the demonstrated absence of mitoses this would rapidly have increased the frequency of nuclei in high DNA classes to unbelievably high levels. The occurrence of DNA synthesis in many nuclei was doubtless brought about by the excision and/or by the culture conditions. However, the frequency of nuclei undergoing DNA synthesis was definitely increased by the added kinetin, more so by IAA, and most effectively by both together. The present results, therefore, confirm our previous findings (PATAU *et al.* 1957) and fit into the scheme we proposed for the role of kinetin-like substances and auxins in the mitotic cycle.

#### Summary

Pith tissue was cultured on modified White's nutrient medium supplemented, except for controls, with 2 mg/l of IAA and/or 0.5 mg/l of kinetin. For autoradiographs sections were used from tissue grown on medium containing tritiated thymidine.

Nuclear DNA contents (Feulgen) were measured by the microspectrophotometric two-wavelengths method. No fading of Feulgen dye in nuclei was found in 11 weeks, in contrast to considerable fading observed in earlier work when a different batch of basic fuchsin had been employed.

Counts of radioactive nuclei in autoradiographs agreed well with microspectrophotometric results on the occurrence of DNA synthesis.

In control cultures, with or without tritiated thymidine, DNA doubling took place in about 20% of the nuclei during the first two days but in few, if any, thereafter.

It was confirmed that kinetin, as well as IAA, increases the frequency of nuclei undergoing DNA synthesis. However, IAA, in contrast with kinetin, still induced considerable DNA doubling after two days. Continued cell reproduction was maintained only in the presence of both substances.

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Dr. N. K. DAS, Dept. of Zoology, University of California,  
Berkeley 4, California, U.S.A.

Dr. K. PATAU, Dept. of Pathology, University of Wisconsin,  
Madison 6, Wisc., U.S.A.

Dr. F. SKOOG, Dept. of Botany, University of Wisconsin,  
Madison 6, Wisc., U.S.A.

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